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University of Louisville, School of Medicine, Louisville, Ky. and University of Michigan Medical Center Ann Arbor Mich.

Absorption and Excretion of Cyanocobalamine after Oral Administration of a Large Dose in Various Conditions[1]

G. RACCUGLIA, A. FRENCH and C. J. D. ZARAFONETIS

The absorption, retention and excretion of vitamin B_{12} after oral intake of physiologic amounts have been exhaustively studied. Less well known is the mechanism of absorption after oral intake of large amounts of this substance. The most important difference is that oral intake of large doses results in absorption of the compound even in absence of intrinsic factor. UNGLEY [1] demonstrated that 5,000 µg of crystalline B_{12} administered orally without intrinsic factor could induce remission of pernicious anemia. Further studies of absorption, excretion and therapeutic response in patients with megaloblastic anemias receiving 1,000 to 5,000 µg of cyanocobalamine *per os* were carried out by CONLEY *et al.* [2], UNGLAUB *et al.* [3] and PITNEY and BEARD [4].

Eliminating the influence of intrinsic factor on vitamin B_{12} utilization, the changes of serum concentrations of the vitamin immediately following its ingestion reflect the combined abilities of the intestine to absorb the compound and of the liver to retain it. If the renal functions are reasonably preserved, the persistence of the vitamin in circulation after absorption and the magnitude of its urinary excretion are mainly functions related to the serum binding capacity for the compound [5, 6, 7] but its retention is also influenced by the degree of tissue saturation [8].

In view of the above facts it was thought of interest to study the serum levels and urinary excretion of vitamin B_{12} after the administration of 3,000 µg *per os* to normal individuals and to patients with

[1] Supported in part by grant No. PHS CA07784.

gastrointestinal defects, pernicious anemia chronic granulocytic leukemia and chronic lymphocytic leukemia. Abnormalities of tissue saturation and serum binding capacity are known to take place in such conditions [7 9]

Since this simple vitamin B_{12} tolerance test indicated that agents different from intrinsic factor may be of significance in regulating the uptake of cyanocobalamine, further investigation of the mechanism of intestinal absorption of physiologic quantities of vitamin B_{12} was carried out in normal individuals and patients suffering from chronic granulocytic leukemia who manifested increased serum avidity for vitamin B_{12}.

Materials and Methods

Vitamin B_{12} was assayed with the microbiological method of HUTNER *et al.* [10] using the Z strain of *Euglena gracilis*. Para-aminobenzoic acid was constantly added to the culture medium.

Collection of specimens was carried out in vitamin free, sterile glassware. Urine specimens were collected without addition of preservative, but the single samples were refrigerated immediately after voiding. The urine specimens were pooled in 24 hour batches, the volume excreted in this period was measured and an aliquot stored at −20°C until the time of assay Venous blood was collected with the same precautions for sterility and contamination of vitamin B_{12}. The patient specimens were allowed to clot at room temperature and the serum separated and stored at −20°C until the time of assay

The test dose of 3,000 µg of cyanocobalamine was administered to the fasting subject early in the morning, after samples of urine and blood for determination of baseline values had been collected for at least 1 full 24 hour period. Blood samples were subsequently obtained 2, 4, 6, 24 and 48 h after the ingestion of vitamin. Breakfast was allowed 2½ h following ingestion of the test dose and meals were given without restrictions thereafter

Fecal excretion of vitamin B_{12} was studied by administering 0.5 µc of ^{60}Co cyanocobalamine, using the commercial material (Abbott) given for the performance of the SCHILLING test. Stools were collected for 6 days following the administration of the test dose, diluted, homogenized in a Waring blender and pooled. The radioactivity contained in an aliquot was measured and the excretion of the compound calculated as percentage of the dose administered.

The tolerance test was performed in 14 normal individuals, 3 patients with untreated and 4 with treated pernicious anemia, 6 patients with untreated and 3 with treated chronic granulocytic leukemia, 3 patients with chronic lymphocytic leukemia, 7 patients with non-tropical sprue, 6 patients with postgastrectomy status. Normal volunteers were medical students and hospital personnel. Eleven were white and 3 were Negro. Their ages ranged between 25 and 57 years. Six were females and 8 were males. The patients with untreated pernicious anemia were diagnosed on the basis of the usual clinical, hematologic and therapeutic criteria. SCHILLING tests were performed in all of these patients after completion of the studies to confirm the diagnosis. The patients with pernicious anemia in remission had SCHILLING test done at least 2 years previous to the study Their therapy consisted of 50 µg of cyanocobalamine every 15 days and the last dose had been administered 2 weeks before the test. The diagnosis of non-tropical sprue with intestinal malabsorption was made on the

basis of clinical evaluation, small bowel biopsy glucose tolerance test, stool fat, stool nitrogen and the vitamin A absorption test. These patients were on no therapy and SCHILLING tests were delayed until the completion of the vitamin B_{12} tolerance test. Gastrectomized patients had either total or sub-total gastrectomy for various primary clinical conditions. The patients were asymptomatic at the time of the test. Patients with active chronic granulocytic leukemia and chronic lymphocytic leukemia had either never been treated or had relapsed after interruption of therapy. Patients with chronic granulocytic leukemia in remission had been treated with busulfan but received no therapy at the time of the test.

Statistical evaluation was carried out by computer center. The significance of differences between normal serum and urinary values and values obtained in patients was calculated by variance analysis[a]

Results

Table I shows the means of the vitamin B_{12} determinations in the normal and patients serum before administration of vitamin B_{12} and at 2, 4 6 24 and 48 h following the administration of the compound. Total excretion of vitamin B_{12} in the urine during the 24 h preceding the test and in the following two 24 hour periods is also indicated.

The retention of vitamin B_{12} when a physiologic quantity was given was estimated in 4 normal individuals and 5 patients with chronic granulocytic leukemia in relapse by calculating the fecal excretion of labelled cyanocobalamine described in "Materials and Methods. These subjects had not undergone the tolerance test and received only approximately 0.5–1 μg of the vitamin, the quantity contained in the dose used in the performance of the SCHILLING test. Four normal subjects retained respectively 93 82, 40 and 71% (mean 71.5%) of the administered radioactive compound. Five patients with chronic granulocytic leukemia retained 26, 0 14 16 and 10% (mean 13.2%) of the same dose of ^{57}Co cyanocobalamine given. The difference was statistically significant at the 0 01 level.

A balance study was carried out in a healthy individual who received 3 000 μg of cold vitamin B_{12} by mouth to which 1 μg of labelled ^{57}Co cyanocobalamine was added. Stools and urine collection and analysis were then carried out as described for the retention of 'physiologic' quantities. The fecal excretion of vitamin B_{12} in 6 day pooled collection was 2.64×10^6 ng (88% of the given dose) The urinary excretion was 195 ng (<0.01) The retention was therefore 12% of which less than 0 1 (1 150 ng) could be accounted for by the plasma level at the highest peak attained.

[a] Dr RONALD KELLAY of Louisville Computer Service kindly performed this analysis.

Table I Mean serum and urinary vitamin B_{12} levels before and after oral dose of 3 000 µg

Type of patient	No. of pa-tients	Serum (in pg/ml) Before	After 2 h	4 h	6 h	24 h	48 h	Urine (in ng/24 h) Before	After 24 h	48 h	Significance of difference of curves from normal (in %) Serum	Urine
Normal	14	490 (SD 223)	748 (SD 311)	785 (SD 339)	722 (SD 334)	580 (SD 272)	504 (SD 191)	120 (SD 77)	368 (SD 199)	178 (SD 150)		
Pernicious anemia in relapse	3	39	373	900	402	317	178	57	264	50	0.1	N.S.
Pernicious anemia in remission	4	163	583	492	480	383	312	118	873	80	0.1	N.S.
Postgastrectomy	6	418	707	927	835	722	722	41	143	85	0.01	0.05
Malabsorption, sprue	7	265	1,314	1 189	923	564	449	39	370	74	0.01	0.01
Chronic lymphocytic leukemia untreated	3	234	464	552	513	366	302	72	140	76	0.05	0.01
Chronic granulocytic leukemia untreated	6	5,180	7 054	7,362	8,061	7,906	7 148	93	132	102	0.01	0.1
Chronic granulocytic leukemia treated	3	2,967	3,767	4 147	3,673	3,625	3,678	191	372	329	0.01	0.05

Discussion

The results of this study suggest that the pattern of absorption and excretion of vitamin B_{12} following the ingestion of a large dose of the substance in normal subjects, consists in a modest increase of serum levels followed by an increament of urinary concentrations. Within 48 h both serum and urinary concentrations return to pre-test values. The balance study shows that 88 % of the orally administered 3 000 μg of the substance is recovered in the stools. Urinary excretion is only a minimal fraction of the substance unaccounted for by fecal excretion. The retained vitamin is also unaccounted for by plasma content at the highest levels observed. The substance, therefore, must be retained by tissues.

In the case of chronic granulocytic leukemia in relapse however the plasma retention is much greater and longer in duration Correspondingly the urinary excretion is substantially less than normal. Hematologic improvement consequent to anti-leukemic therapy is associated with a marked decrease of plasma retention of vitamin B_{12} [6 7] In view of these facts the B_{12} metabolic pattern in chronic granulocytic leukemia is one of 'high plasma avidity for that compound

In patients with chronic lymphocytic leukemia circulating vitamin B_{12} reached levels which are lower than normal and urinary excretion was less than in normal subjects. This suggests decreased intestinal absorption of vitamin perhaps related to the lower binding capacity of plasma for vitamin B_{12} which these patients manifest [7] The metabolic pattern of these individuals in one of 'low plasma avidity

In pernicious anemia in relapse the plasma increment is very substantial, the return of plasma levels to low values is rapid, and the urinary excretion after absorption modest. This suggests rapid retention of the vitamin by tissues. The metabolic pattern of these patients is one of 'high tissue avidity' Patients with treated pernicious anemia, in contrast, manifest modest plasma increments and urinary excretion twice the normal and three times that of patients in relapse The metabolic pattern is that which can be expected in case of 'low tissue avidity' in these patients receiving vitamin B_{12} parenterally in large quantities.

Patients suffering with intestinal malabsorption manifest a pattern similar to that of pernicious anemia in relapse but exaggerated,

Table 1 Mean serum and urinary vitamin B_{12} levels before and after oral dose of 3,000 μg

Type of patient	No. of patient	Serum (in pg/ml) Before	After 2 h	4 h	6 h	24 h	48 h	Urine (in ng/24 h) Before	After 24 h	48 h	Significance of difference of curves from normal (in %) Serum	Urine
Normal	14	490 (SD 223)	748 (SD 311)	783 (SD 330)	722 (SP 334)	580 (SD 272)	501 (SD 191)	120 (SD 77)	368 (SD 199)	178 (SD 150)		
Pernicious anemia in relapse	3	99	373	900	402	317	178	57	261	50	0.1	N.S.
Pernicious anemia in remission	4	163	583	492	480	383	312	118	873	80	0.1	N.S.
Postgastrectomy	6	418	707	927	833	722	722	41	143	85	0.01	0.05
Malabsorption, sprue	7	263	1,314	1,180	923	561	449	39	370	74	0.01	0.01
Chronic lymphocytic leukemia untreated	3	231	461	552	513	368	302	72	140	76	0.05	0.01
Chronic granulocytic leukemia untreated	6	5,180	7,051	7,362	8,001	7,908	7 148	93	132	102	0.01	0.1
Chronic granulocytic leukemia treated	3	2,967	3,767	4 147	3,673	3,625	3,678	101	372	329	0.01	0.05

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Authors' addresses: Dr. GIOVANNI RACCUGLIA, University of Louisville, School of Medicine, *Louisville, Ky.* Dr. A. FRENCH and Dr. C. J. D. ZARAFONETIS, University of Michigan Medical Center *Ann Arbor, Mich.* (USA).

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Departments of Medicine A and B, Hadassah University Hospital and Medical School, Jerusalem

Studies in Cryofibrinogenemia[1]

A. Zlotnick, W. Shahin[2] and E. A. Rachmilewitz

A cold-precipitable protein was first described in a case of multiple myeloma by Wintrobe and Buell in 1933 [1] and named cryoglobulin by Lerner and Watson in 1947 [2]. The cryoglobulins are mostly γG (7 S) globulins or γM (19 S) globulins, which precipitate upon cooling of the serum. In 1956 Korst and Kratochvil [3] observed in a patient with carcinoma of the lung associated with thrombophlebitis migrans, a protein which precipitated on cooling of the plasma but not of the serum. They called this protein cryofibrinogen. Cryofibrinogenemia was found to be associated with acute infections [4], metastatic carcinoma, leukemia [5] and pregnancy [6]. Essential cryofibrinogenemia, in which no underlying disease is present, is apparently rare, as only a few cases have been reported [7, 8, 9].

The purpose of the present study was to determine the incidence of cryofibrinogenemia in patients with a highly increased sedimentation rate, and to compare the composition of the cryoprecipitate of heparinized and oxalated plasmas, using immunochemical methods. This study reports the finding of a cryofibrinogen in 71 patients and of a cryoglobulin in 9 patients out of a total of 89 patients with an increased sedimentation rate examined consecutively. The splitting of the fibrinogen molecule into two fractions having different mobilities, as was observed with the oxalated cryoprecipitate in a previous work [10] did not occur in the cryoprecipitate obtained from heparinized plasma. Evidence is presented that plasmin splits the fibrinogen in the cryoprecipitate obtained from oxalated plasma.

[1] This work has been supported in part by a grant from the Israel Cancer Association.

[2] Part of this work was submitted as an M.D. thesis.

Material and Methods

Eighty-nine patients with an increased sedimentation rate ranging from 45 to 99 mm in the first hour and 100 to 165 mm in the second hour were the subjects of this study. Twenty-two medical students and interns with a normal sedimentation rate (mean 8/16 mm) served as controls.

Blood was collected from an antecubital vein, and 10 ml were put into each of 4 test tubes. The first did not contain any anticoagulant, the second contained 1 mg heparin, the third 2 mg heparin and the fourth 20 mg of dry ammonium-potassium oxalate. The serum, heparinized and oxalated plasmas obtained were left for 48 h at 4° C. The cryoprecipitate was separated from the supernatant by centrifugation in a refrigerated centrifuge at 4,000 rpm for 20 min and washed twice in cold buffered saline, pH 7.4. It was resuspended in 1–2 ml of saline and dissolved by incubating for 12 h at 37° C.

Total serum protein was determined by WEICHSELBAUM's method [11]. Fibrinogen was determined in the oxalated plasma, in the redissolved cryoprecipitate and in the supernatant plasma according to RATNOFF and MENZIE [12].

Paper electrophoresis of the serum, plasma, and of the cryoprecipitate was performed with a Spinco model R apparatus using veronal buffer pH 8.6, ionic strength 0.1. The strips were heat fixed and stained by amido B. black and were scanned in a Spinco Analytrol.

Immunoelectrophoresis was performed with the micro-apparatus of SCHEIDEGGER [13] using 25 × 75 mm microscope slides. A 2 percent agar solution in sodium barbital buffer pH 8.2, ionic strength 0.04, was used as a medium for electrophoretic separation.

Antisera. Three different antisera were used: 1. Hyland IEP horse anti-human total antiserum lot No. 5803. This antiserum was found to contain antibodies also to fibrinogen. 2. Rabbit anti-human fibrinogen serum, Hyland RP 4-760. 3. Rabbit anti-cryofibrinogen serum, obtained by immunizing rabbits with the dissolved oxalated cryoprecipitate of a patient with cryofibrinogenemia. Four injections of 10 mg each of cryoprecipitate protein, incorporated in complete Freund's adjuvant, were given subcutaneously to 4 rabbits at weekly intervals. Ten days after the last injection, the animals were bled and their serum was absorbed with different amounts of normal human serum, until a monovalent anti-cryofibrinogen serum was obtained.

Results

Table I indicates that out of 89 patients with an increased sedimentation rate, 71 had a cryoprecipitate in the plasma only, 9 had it both in the serum and the plasma and 9 did not show any cryoprecipitate. The first group was considered to have cryofibrinogenemia and the second, cryoglobulinemia. Eight patients in the first group and 5 in the second showed signs and symptoms which could be attributed to the presence of a cryoprecipitate. These were: cutis marmorata, RAYNAUD's phenomena, acrocyanosis, skin hemorrhages or necrosis of the extremities. Table II shows the different diseases in which cryoprecipitates were found in the plasma.

The present study deals with the cases of cryofibrinogenemia only; the cases of cryoglobulinemia will be reported subsequently. Table III

Table I Incidence of cold-precipitable protein among patients with raised sedimentation rate

	No. of patients	Asymptomatic	Symptomatic[1]
Cryofibrinogenemia	71	63	8
Cryoglobulinemia	9	5	4
Without cryoprecipitate	9	–	–
Total	89	68	12

[1] Cutis marmorata, Raynaud phenomena, acrocyanosis, necrosis of extremities, hemorrhages.

Table II Association of cryofibrinogenemia with various disorders

	Total No.	Symptomatic
Essential	3	3
Infection	17	
Metastatic carcinoma	14	1
Macroglobulinemia of WALDENSTRÖM	2	
Chronic lymphatic leukemia with macroglobulinemia	1	
HODGKIN's disease	2	
Myocardial infarction	4	1
Collagen disease	11	3
Miscellaneous[1]	17	
Total	71	8

[1] Familial mediterranean fever, amyloidosis, thyroiditis, bone fracture, diabetes mellitus.

shows the sedimentation rate, the fibrinogen level, the serum globulins and the protein content of the cryoprecipitate of 60 patients with cryofibrinogenemia, and of 22 normal control subjects. In the controls, who had a normal sedimentation rate, no cryoprecipitate was observed in the oxalated plasmas and in the plasmas which contained 2 mg/ml of heparin. Only unmeasurable traces of a cryoprecipitate were seen in the plasmas which contained 1 mg/ml of heparin. The concentrations of serum proteins and of the plasma fibrinogen were within normal limits.

Table III Sedimentation rate (1/2 h) fibrinogen level, serum globulins and protein content of the cryoprecipitate in patients with cryofibrinogenemia

Group	No. of cases		Sedimentation rate, mm	Fibrinogen mg%	α_2-Globulin g% TP	γ-Globulin g% TP	Pp oxalat mg%	Pp heparin I mg%	Pp heparin II mg%
A	36	M	100–165	680 ± 207	1.19 ± 0.23	1.75 ± 0.35	101 + 71	228 ± 109	172 ± 93
		R		(429–1100)	(0.76–1.89)	(0.79–2.84)	(18–267)	(49–450)	(31 399)
	8	M	100–145	354 ± 53	1.14 ± 0.18	1.71 ± 0.64	70 ± 27	145 ± 33	80 ± 27
		R		(284–398)	(0.81 1.45)	(0.81 2.59)	(36–128)	(100–195)	(50–131)
B	10	M	65–99	583 ± 115	1.11 ± 0.22	1.85 ± 0.32	93 ± 64	182 ± 46	127 ± 91
		R		(410–869)	(0.91 1.36)	(1.22 2.47)	(23–253)	(20–392)	(51 290)
	6	M	43–98	327 ± 69	1.20 ± 0.23	2.41 ± 0.54	79 ± 35	144 ± 40	102 ± 31
		R		(209–400)	(0.95–1.35)	(1.01 2.51)	(20–121)	(106–239)	(71 153)
Control	22	M	8–16	305	0.63	1.54	0	Traces	0
		R		(200–400)	(0.48–0.80)	(1.40–1.73)			

A = Patients with sedimentation rate above 100
B = Patients with sedimentation rate below 100
M = Mean and standard deviation
R = Ranges of experimental values
TP = Total proteins

Pp oxalate = Cryoprecipitate of oxalated plasma
Pp heparin I = Cryoprecipitate of blood containing 0.1 mg% of heparin
Pp heparin II = Cryoprecipitate of blood containing 0.2 mg% of heparin

The patients with cryofibrinogenemia were divided into two main groups: those with a sedimentation rate of above and below 100 mm (groups A and B respectively) in the second hour. In 8 out of 44 patients in group A, the plasma fibrinogen level was within normal limits, in the remaining 36, the plasma fibrinogen was elevated. A cryoprecipitate was found in both of these subgroups. There was no correlation between the fibrinogen level of the plasma or the concentration of the serum proteins and the amount of the protein in the plasma cryoprecipitates. Sometimes patients with a high plasma fibrinogen showed less cryofibrinogen than patients with a normal plasma fibrinogen. In all of the cases, the heparinized plasma cryoprecipitate had a higher protein content than the oxalated plasma cryoprecipitate. However an increase of the heparin in the plasma from 0.1 to 0.2 mg/ml caused

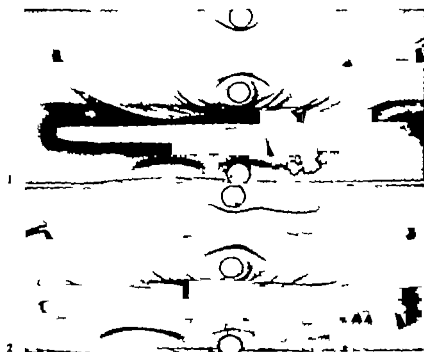


Fig. 1. Immunoelectrophoretic pattern of the plasma (central well) and the heparinized cryoprecipitate (outer wells) run against a polyvalent antiserum (lower trough) and an antifibrinogen serum (upper trough). Note the normal position of the fibrinogen arc.

Fig. 2. The same patient and the same arrangement as in figure 1 except that the outer wells contain the oxalated cryoprecipitate. Note the double curvature of the fibrinogen arc.



Fig. 3. Immunoelectrophoretic pattern of cryoprecipitate obtained from oxalated plasma, run against an antifibrinogen serum (upper trough) and polyvalent antiserum (lower trough). Note the two fibrinogen arcs and the presence of other proteins in the cryoprecipitate

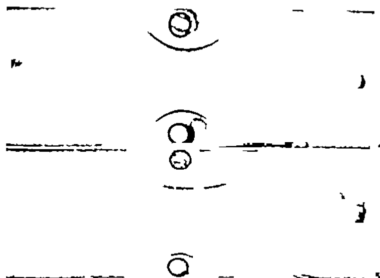


Fig. 4. Immunoelectrophoretic tracing of heparinized cryoprecipitate with epsilon aminocaproic acid (lower well and without it (upper well) run against an antifibrinogen serum.

Fig. 5. The same arrangement as in figure 4, except that the cryoprecipitate was obtained from oxalated plasma. Note the change in mobility of the fibrinogen arc of the cryoprecipitate without epsilon aminocaproic acid.

a diminution of the cryoprecipitate. Similar results were obtained with 16 patients with cryofibrinogenemia whose sedimentation rate was below 100 mm for the second hour (group B)

Immunoelectrophoresis (IEP) was performed on plasma and on redissolved oxalated – and heparinized – plasma cryoprecipitates (fig 1 and 2) In both oxalated and heparinized plasmas the fibrinogen appeared as a single band near the antigen well. However in the cryoprecipitates there was a difference whereas in heparinized cryoprecipitates the fibrinogen appeared also as a single precipitin band (fig 1) it appeared in oxalated cryoprecipitates either as a double curved band (fig 2) or as two bands of different mobilities (fig 3) In some instances, the fibrinogen appeared as a single band but its mobility differed from that of normal fibrinogen. The dissolved plasma cryoprecipitate run against a polyvalent antiserum showed that besides the cryofibrinogen other proteins were also present in the cryoprecipitates (fig 1 2, 3) The form or intensity of the fibrinogen band of plasma or of the cryoprecipitates did not differ when the anticryofibrinogen antiserum was substituted for the commercial antifibrinogen antiserum.

When epsilon aminocaproic acid (EACA) in a concentration of 50 mg was added to heparinized or oxalated plasma the formation of a cryoprecipitate was not inhibited When these cryoprecipitates were dissolved and examined by immunoelectrophoresis against a monovalent antifibrinogen antiserum, there was no difference in form or mobility of the cryofibrinogens in the cryoprecipitate from heparinized plasma either with or without EACA (fig 4) However IEP of the oxalated plasma cryoprecipitate to which EACA was added did not show the splitting or any change in mobility of the fibrinogen precipitin arc as seen in the oxalated plasma cryoprecipitate which had formed without EACA (fig 5) IEP revealed more precipitation bands in the heparin cryoprecipitate than in the oxalate cryoprecipitate

Discussion

The present study indicates that cryofibrinogenemia occurs more frequently than cryoglobulinemia. Whereas cryoglobulinemia is mainly associated with lymphoproliferative disorders, cryofibrinogenemia is mostly associated with processes of tissue destruction such as acute infections, metastatic carcinoma, myocardial infarction and pregnancy though it can also be found in macroglobulinemia and collagen disorders reputed for the proliferation of their lympho-plasma cellular series

It is apparent that there is no correlation between the plasma fibrinogen level and the amount of cryoprecipitate formed for the plasmas of some patients with normal fibrinogen levels showed heavier cryoprecipitates than those of other patients with increased fibrinogen levels. A similar observation was reported by McKEE *et al.* [5] who found cryofibrinogens at all ranges of plasma fibrinogen levels in 22 out of their 665 hospitalized patients. The only possible deduction from our results is that the higher the sedimentation rate the heavier is the plasma cryoprecipitate.

In all our cases the cryoprecipitate of the heparinized plasma was heavier than that of the oxalated plasma. IEP of these cryoprecipitates showed more precipitation bands in the heparin-cryoprecipitate than in the oxalate-cryoprecipitate SMITH *et al.* [4] showed that cryofibrinogen occurs more frequently and in higher concentrations when heparin is used as an anticoagulant. Their optimal concentration was 0 1 to 0 15 mg/ml of heparin. We confirmed this observation, for when the heparin concentration was increased from 0 1 to 0 2 mg/ml, the amount of cryoprecipitate diminished

In a previous publication [10] we reported that the fibrinogen precipitation band obtained by immunoelectrophoresis of the dissolved cryoprecipitate formed from oxalated plasma was split into two arcs of different mobilities, whereas the fibrinogen in oxalated plasma incubated at 37°C for an identical period showed a single precipitin arc near the antigen well. In the present work it has been shown that the fibrinogen band of the dissolved cryoprecipitate formed from heparinized plasma did not split and remained at the same place as the plasma fibrinogen. Since heparin is known to inhibit the transformation of plasminogen into plasmin [14] it was assumed that the splitting of the fibrinogen in the oxalate-cryoprecipitate was due to plasmin activity This hypothesis was tested by adding epsilon aminocaproic acid to oxalated plasma to inhibit plasmin activity When IEP was performed with the dissolved cryoprecipitate from EACA-treated plasma, the fibrinogen band was not split. This provided evidence suggesting that the splitting was due to plasmin activity in the cryoprecipitate.

The mechanism of the precipitation of the cryofibrinogen was not elucidated in this study Though the cryoprecipitate was washed thoroughly it still contained other proteins besides the fibrinogen. The role of these proteins in the cryoprecipitation process is not clear Whether they are an active factor in the process or whether they

coprecipitate passively with the fibrinogen remains to be established. The fact that an identical precipitation reaction was obtained with the anticryofibrinogen serum and with the antinormal fibrinogen acting against the fibrinogen in the cryoprecipitate, indicates that the cryoprecipitation did not change the antigenicity of the fibrinogen. Thus if there is a structural change in the fibrinogen molecule on precipitation in the cold, it cannot be detected by immunochemical methods.

Cryofibrinogenemia is sometimes associated with thrombohemorrhagic and Raynaud-like phenomena. In some cases [15] it was shown that the cryofibrinogen formed complexes with clotting factors, thus explaining the hemorrhagic phenomena. Since cryofibrinogenemia is associated with processes of tissue destruction, we may assume that the blood of these patients contains intracellular enzymes, some of which have a proteolytic activity and produce vasoactive polypeptides, which may cause the Raynaud like phenomena.

Summary

In [illegible] patients with increased sedimentation rates cryofibrinogen was found in [illegible] and cryoglobulin in [illegible]. In the dissolved cryofibrinogen other plasma proteins were also present. Immunoelectrophoresis of the dissolved cryoprecipitate obtained from heparinized plasma showed a single precipitation band, but two bands were observed when the cryoprecipitate had been obtained from oxalated plasma. The splitting was caused by the action of plasmin on fibrinogen, because it could be prevented by heparin and by epsilon aminocaproic acid. It is suggested that cryofibrinogenemia is associated with processes that liberate intracellular enzymes.

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Authors' address: Prof. Dr. A. Zlotnick, Dr. W. Shaked and Dr. E. A. Rachmilewitz, Department of Medicine, Hadassah University Hospital and Medical School, Jerusalem (Israel)

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Department of Laboratory Haematology, University of Toronto Clinic, Sunnybrook Hospital, Toronto

Leukocyte Alkaline Phosphatase

The Effect of Age and Sex

P. K. Ray and P. H. Pinkerton

The histochemical assessment of leukocyte (neutrophil) alkaline phosphatase (LAP) activity has been used for some time in the laboratory diagnosis of myeloproliferative disease. Low levels of enzyme activity are characteristically found in chronic myeloid leukaemia and in myeloblastic leukaemia in relapse; normal or elevated levels are generally found in association with other leukaemias, leukaemoid reactions, polycythaemia vera and reactive leukocytosis [1–9].

Fluctuations in LAP activity are seen during the menstrual cycle [10, 11] and both pregnancy and the use of oral contraceptives raise the LAP level [10, 12–16]. Using an enzyme assay method, Rosner and Lee [17] demonstrated differences in LAP activity between males and females, and between pre-menopausal and post-menopausal females. Employing a histochemical assessment of LAP activity, we have noted similar differences between males and females and have also observed a significant decrease in LAP activity with ageing in both sexes.

Materials and Methods

559 males and 227 females, aged between 15 and 95 years were studied. The majority were blood donors. The remainder were healthy hospital employees, convalescent patients recovering from fractures and orthopaedic procedures, but otherwise well, patients in chronic care wards and attending a geriatric clinic, but believed not to be suffering from neoplastic or inflammatory disorders. All showed morphologically normal blood smears. None of the female subjects admitted to being pregnant at the time of study and it is not known how many were taking oral contraceptives.

The staining technique was modified from that of Hayhoe et al. [18]; blood films were exposed to substrate at 37°C for 40 min, instead of incubating at room temperature for 8–10 min. The smears were washed, counterstained with 0.5% methyl green, rinsed, mounted and scored according to standard criteria [18]. In most instances 100 cells, and, in the remainder 50 cells were scored, and the results expressed as total 'LAP score' per 100 neutrophils, the 'LAP score' may thus be between zero and 400.

Results

The results are presented in figure 1. The LAP score is significantly higher in females than in males up to the age of 50 years; thereafter there is no significant difference between the sexes.

Statistical analysis of the age changes in LAP score shows a highly significant decline in enzyme activity after the 7th decade in men ($p < 0.001$) and after the sixth decade in women ($p < 0.001$). The fall in LAP activity after the age of 60 years is somewhat sharper in women than in men. Significantly lower LAP scores are found in youths in the

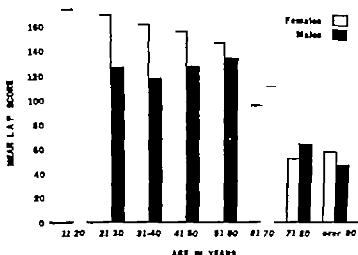


Fig. 1. The changes in the mean leukocyte alkaline phosphatase score (LAP score) with age and sex. Stippled columns represent females and the closed columns, males. All subjects in the second decade (11–20 years) were 15 years of age or over. When the LAP scores for males and females are compared for the second to fifth decades, the differences are significant (second to fourth decades, $p < 0.001$; fifth decade $p < 0.005$). Thereafter no significant difference is seen between the sexes ($p > 0.5$).

second decade than in men in the third to sixth decades (p <0 01) By contrast, females in the second decade show the highest LAP scores.

In some healthy subjects over 70 years of age, LAP scores in the range 14–20 were found, and in two patients, over 70 years of age, with untreated chronic myeloid leukaemia scores between 0 and 18 have been observed. In two other cases of chronic myeloid leukaemia, in a younger group scores between 2 and 14 were found. In spite of the wide range of normal, cases of polycythemia vera (3) disseminated carcinomatous (3) multiple myeloma (6) and reactive neutrophil leucocytosis (8) have all shown high LAP activity with scores in the range 200–3 0

Discussion

By the modified technique used here, the normal range for the LAP score tends to be higher than reported previously [4 5 19–21] Precipitation of substrate is increased by the more prolonged incubation at 37 C, and it may be that this heavier precipitation has emphasized the differences in LAP score with age and sex, differences wich have not hitherto been appreciated.

The changes in LAP activity with the menstrual cycle, pregnancy and the use of oral contraceptives (as mentioned above) and the increase in LAP activity produced by pituitary and adrenal hormones in man [22] and by oestrogens, progesterones and testosterone in experimental animals [23] imply that hormonal factors influence the LAP The variations with age and sex in LAP score reported here confirm the differences previously described employing an enzyme assay technique [17] and are in keeping with the hypothesis that the level of LAP activity is at least partly controlled by the sex hormones. The prevalence of oral contraception among the women studied here is not known. However it seems unlikely that these agents alone would be responsible for the difference observed between the sexes in the younger age groups i.e. under 50 years) and especially in the 20 years and under group where the sex difference in LAP score is most obvious.

Levels of LAP activity have long been known to be low in chronic myeloid leukaemia [1 2, 4–6] and the LAP score is occasionally employed in the laboratory diagnosis of this disorder particularly in atypical cases where diagnosis is difficult. This study indicates that a

low LAP score may be a manifestation of ageing and caution should be exercised in the interpretation of the result of this investigation in the elderly

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Summary

The leukocyte (neutrophil) alkaline phosphatase (LAP) has been determined in 380 males and 227 females, using histochemical method. Activity of this enzyme is significantly higher in females than in males from the second to the fifth decades; thereafter no significant difference is noted. In both the LAP score declines with age, and, in the elderly (over 70 years) overlap with patients of similar age with chronic myeloid leukaemia may be seen. The need for caution in interpreting low LAP scores in suspected chronic myeloid leukaemia in the elderly is stressed.

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injected into baby mice a marked lymphocytosis was observed the degree of which correlated fairly well with the clinical assessment of the activity of the disorder. The effect reached its maximum intensity after 6 days and disappeared after 14 days. A search was made [2, 3] for LSF in a number of other disorders including other forms of leukaemia. It was found only in CLL, lymphosarcoma and myelofibrosis.

Evidence for the presence of a feedback mechanism can be found in the work on LAP rats [1]. LAP treatment of the rats 8 times removed 50 more leucocytes than LAP treatment 6 times. This suggests that the leucocyte depletion had already begun to cause an increased LIF production. A lymphocytosis stimulating substance (LSS) has been found in both mouse and human thymus [4]. Both LSS and LSF have been shown to produce a lymphocytosis in baby mice and at the same time to inhibit LSS production in these mice [4]. This suggests a negative feedback mechanism is controlling LSS production.

Evidence is available from the literature, therefore, that humoral substances can be found in both animals and man which affect the numbers of circulating lymphocytes. For the hypothesis presented here it is necessary to show that lymphopoietin can cause an increase in the rate of mitosis. If the hypothesis is correct the serum of patients with CLL should contain a relatively large amount of lymphopoietin. This has been investigated by adding CLL serum to phytohaemagglutinin (PHA)-stimulated normal human lymphocytes in culture.

Materials and Methods

The CLL serum used was prepared as follows. After diagnosis but before treatment venous blood was obtained and placed in sterile bottles and allowed to clot. The serum was removed and spun down at 3 000 rpm for 15 min to remove any cells present. The supernatant serum was then kept frozen at -20° C until used.

The cultures were set up as follows. Twenty ml of venous blood was obtained from healthy volunteers and immediately transferred to a sterile bottle containing anticoagulant (heparin in dextran). After allowing the erythrocytes to settle the supernatant plasma and cells were withdrawn. Each culture contained 1.5 ml of plasma and cells. The experimental cultures had 2.0, 1.0, 0.5 or 0.2 ml of CLL serum added. All cultures were made up to a final volume of 10 ml with TC 199 culture medium. Experimental cultures were set up both with PHA, two drops of reconstituted PHA per culture and without PHA. All cultures were incubated for 72 h at 37° C at which time 0.2 ml of demecolcine (Colcemid®) at a concentration of 1 mg in 100 ml was added and the cultures were incubated at 37° C for a further 2 hours. The cells were then spun down at 1 000 rpm for 10 min, resuspended in 3 ml of hypotonic saline for 15 min at 37° C and then fixed in acetic acid/alcohol (1:3) over-

night. They were then spun down and resuspended in 45% acetic acid, spread onto cold slides, air dried and stained with 10% Giemsa in pH 6.4 buffer. The mitotic index, here defined as the number of mitoses per 1,000 cells was determined by examination of at least 1,000 cells from each culture. The experiment was performed in triplicate.

Results

The average results of the experiments using PHA are given in table I and are expressed in the third column as a percentage of the control value. It will be seen that there was a definite and significant increase in the rate of mitosis in the cultures treated with CLL serum. In the cultures without PHA no transformation of the lymphocytes took place showing that the effect of the CLL serum was on the transformed cells capable of mitosis and that the increase in mitotic rate found was not due to antigenic stimulation of the lymphocytes by CLL serum.

Discussion

The suggestion made here is that LIF LSF LSS and the mitotic stimulator found in CLL serum are all lymphopoietin It has been shown that humoral substances which increase the rate of lymphocyte production are formed in conditions of leucopenia [1] and are present in cases of lymphocytosis [2 3] and are found in thymus [4] It is further shown here that at least one of these substances acts as a mitotic stimulator and thus all parts of the hypothesis have some supporting experimental evidence.

Table I. The effect of CLL serum on the mitotic index of normal human PHA-stimulated lymphocytes in culture (average of 3 experiments)

CLL serum per culture, ml	Mitoses per 1,000 cells	Percent of control
0.2	28	373.3
0.5	27	360.0
1.0	16.5	220.0
2.0	10	133.3
0.0 (control)	7.5	100.0

It need hardly be emphasised that this hypothesis, if correct, raises a number of interesting questions. If, as a number of workers believe, there is more than one population of lymphocytes in the body does lymphopoietin occur in more than one form? Even more important is the question of the site of lymphopoietin production. From the available evidence [4] the thymus seems the most probable and this organ has long been implicated in lymphocyte production. If this hypothesis is supported by further investigations then different approaches to the treatment of lymphoproliferative disorders may become possible.

Acknowledgement. I wish to thank the Smith Kline and French Foundation for financial support.

Summary

Evidence is presented for the hypothesis that a humoral substance, lymphopoietin, which acts by controlling the rate of mitosis in the lymphocyte production pathway controls lymphopoiesis. It is suggested that the amount of lymphopoietin produced in the body possibly in the thymus, is controlled by a negative feedback mechanism and that a lymphocytosis may occur when this feedback mechanism fails.

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Author's address: Dr J. Timson, University Department of Medical Genetics, The Royal Infirmary, Manchester 13 (United Kingdom)

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Institut d'Hématologie, Service des Maladies du Sang et Centre Régional de Transfusion Sanguine de Strasbourg (Prof. R. Waitz)

Le rôle des inhibiteurs faibles du facteur VIII

G. Mayer, Geneviève Noel et R. Waitz

avec l'assistance technique de Marlene [illegible]

Nous avons relevé dans un ensemble de cas cliniques qui nous étaient adressés pour un syndrome hémorragique fruste peu inquiétant, ou pour une simple anomalie de la crase sanguine constatée dans d'autres laboratoires, une activité neutralisante faible mais incontestable du plasma vis-à-vis du facteur VIII. Comme le système des inhibiteurs de la coagulation reste assez mal connu et sa pathologie est plus étendue qu'elle n'apparaît aujourd'hui sur la foi d'exemples frappants mais très rares pour le praticien, nous avons cru utile de publier ces quelques observations. Ce sont des cas de routine qui concernent le plus souvent de petits malades, difficiles à tenir sous une analyse exhaustive et même des bien portants.

Matériels et méthodes

Pour les techniques de routine nous renvoyons aux ouvrages qui les exposent [4, 7]. La consommation de la prothrombine est donnée par la prothrombine résiduelle vraie du sérum laissé quatre heures à 37° C. L'effet inhibiteur d'un plasma sur la mixture normale du test de génération de la thromboplastine (TGT) se manifeste lorsque ce plasma sert de substrat dans ce test.

Recherche d'un inhibiteur du facteur VIII. Du plasma citraté est agité deux fois pendant 20 min à la température du laboratoire avec 100 mg/ml de sulfate de baryum jusqu'à l'obtention d'un temps de Quick supérieur à 15 min (plasma Ba). Ce plasma Ba citraté que nous utilisons pour le TGT depuis plus de dix ans conserve mieux et plus longtemps son facteur VIII et son facteur V que le plasma Ba oxalaté. Le sérum provient de sang recueilli sur thromboplastine cérébrale humaine et laissé quatre heures à 37° C.

Un volume de plasma Ba à tester non dilué (ou parfois de sérum ou de fraction à tester) est incubé pendant 60 min (initialement 30 min) à 37° C avec 4 volumes d'un pool entier de plasmas Ba normaux, source de facteur VIII. Un autre mélange, où le tampon barbital-saline remplace le matériel à tester est mis en route parallèlement. A la fin de

l'incubation les mélanges sont dilués au 1:10e en imidazol-saline. Le facteur VIII résiduel est déterminé dans le TGT.

Dans ce but nous y avons aménagé de légers changements de technique et une importante modification de lecture. Dans la mixture le plasma Ba est introduit dilué 1:10, le sérum 1:20. L'étalonnage est fait à 20, 10, 5 et 2,5% de plasma Ba normal dans le plasma Ba hémophile; les valeurs sont reportées sur papier bilogarithmique. Le matériel à tester 1:10 remplace ensuite à 20% dans le plasma Ba hémophile le plasma Ba normal. Son taux de facteur VIII se lit alors aisément sur la courbe d'étalonnage (= valeur lue × 5).

Le paramètre que nous utilisons est le temps de coagulation minimum (TCm) de chaque mixture testée dans le TGT. Nous ne définissons jamais un point chronologique fixe où tester toutes nos mixtures, par exemple le moment du TCm d'une mixture normale. Au contraire, nous suivons chaque mixture pas à pas jusqu'à ce qu'elle ait passé son TCm. Le TGT est très complexe. Il renferme beaucoup d'éléments certainement très intéressants mais inconnus et non maniables. Le TCm seul est un paramètre remarquable. Il est stable, c'est-à-dire qu'il reste toujours dans le même rapport quantitatif avec les TCm des témoins. Il est sensible, car il se modifie proportionnellement aux corrections et de manière à engendrer une ligne droite sur un papier bilogarithmique. Il définit la mixture qui le donne. Par contre le moment où il se produit est très variable selon des circonstances que nous saisissons mal. Nous avons bien souvent pu constater que pour un temps d'incubation strictement défini, les TC des diverses mixtures subissent une distorsion et ne sont plus vraiment proportionnels entre eux. Aussi ne nous paraissent-ils plus caractéristiques et préférons nous au prix d'un travail plus long, déterminer ce TCm qui n'est pas simplement un TC parmi d'autres, le plus faible, mais aussi un paramètre de signification particulière.

L'unité d'inhibiteur du facteur VII sera, selon BIGGS *et al.* [2, 15], la quantité capable de neutraliser 75% du facteur VIII contenu dans un ml de plasma.

Compte tenu de ce qui précède, la concentration I d'inhibiteur exprimée en unités/ml sera donnée par la formule

$$I = \frac{\left(100 - 100\frac{C}{Co}\right) \times 5}{75}$$

où C est le taux du facteur VIII résiduel du mélange à tester, Co celui du mélange témoin à 20% de tampon représentant la valeur initiale du facteur VIII, 100 C/Co le taux du facteur VIII résiduel dans le mélange à tester exprimé en % de la valeur initiale et 100–100 C/Co le facteur VIII disparu en % de la valeur initiale.

Un manque de 15% du facteur VIII initial répond à l'unité d'inhibiteur mais ce mouvement est trop faible pour se dégager des erreurs d'appréciation. Nous estimons que l'inhibiteur n'est certain que pour les déficits après incubation supérieurs à 30%, soit 2 unités. Nous retenons comme douteuses les valeurs entre 1,6 et 2 unités.

La recherche d'un inhibiteur du facteur IX répond aux mêmes principes. Mais elle se fera sur le sérum incubé avec un pool de sérums normaux, le mélange étant ensuite dilué 1:20 et testé sur le sérum hémophile B dans le TGT. L'unité d'inhibiteur pourra être définie de manière analogue.

Résultats

Circonstances étiologiques (tableaux I et IV). Les femmes prédominent parmi nos malades ainsi que les sujets jeunes. Les deux tiers en ont moins de 20 ans, aucun n'atteint la soixantaine.

Tableau I Données cliniques et étiologiques

Cas Nº	Sexe	Age ans	Affection	Syndrome hémorragique	Données étiologiques	Hérédité	Remarques
1	M	19	épistaxis	discret épistaxis	transfusions	0	
2	F	11	hémarthrose	discret hémarthrose	0	0	
3	F	23	syndrome hémorragique	franc coupures, avulsions dentaires, gingivorragies, épistaxis, ecchymoses, postpartum	2 grossesses (2e interrompue) transfusions	hémorragies chez le grand-père et l'arrière grandpère paternel et chez la fille. Inhibiteur du facteur VIII chez la cousine maternelle (Cas N 5)	maladie mitrale post-rhumatismale
4	F	34	métrorragies	franc coupures, interventions, gingivorragies, épistaxis, métrorragies, postpartum	4 grossesses transfusions	hémorragies chez 2 des 4 garçons, mais absence d'inhibiteur du facteur VIII	œdème de Quinke
5	F	18	–	0	0	cousine maternelle du cas Nº 3	
6	M	9	maladie de von Willebrand	franc (modéré)	0	?	
7	F	10	métrorragies	franc coupures, avulsions dentaires, interventions, gingivorragies, métrorragies	0	hémorragies chez la mère et les deux sœurs	
8	F	53	syndrome hémorragique	franc coupures, vulsions dentaires, gingivorragies, épistaxis, ecchymoses, pétéchies	5 grossesses	hémorragies chez les 3 filles et 1 des 2 garçons mais absence d'inhibiteur du facteur VIII	séquelles de phlébites post-gravidiques

Tableau I (suite)

Obs Nº	Sex	Age ans	Affection	Syndrome hémorragique	Données étiologiques	Hérédité	Remarques
9	F	56	hépatosplénomégalie chronique	0	transfusions	0	
10	M	14	splénomégalie bronique	fracas épistaxis, hématomes, coupures purpura pétéchal des membres inférieurs	transfusions	hémorragies chez oncle maternel	fibrosplénie hémolyse
11	M	19	abcès sous-phrénique droit	0	transfusions	0	séquelles d'hépatite virale
12	M	33	gammamyélome hépatite d'inoculation	0	transfusions	0	nécrose hépatique traumatique résection hépatique partielle
13	F	19	rthralgies, douleurs abdominales fièvre	discrets rectorragies ménorragies	pénicilline	0	
14	F	7	végétations adénoïdes	discrets ecchymoses	0	0	angines phlegmoneuses répétées

Un grand nombre avait reçu des transfusions de sang et un au moins de la pénicilline, avant que l'inhibiteur ait été découvert. De même, près de la moitié des femmes avaient eu des grossesses dont plusieurs multiples et les incidents de la délivrance avaient été souvent l'occasion de transfusions. L'effet d'une grossesse associé à celui des transfusions apparaît de manière frappante dans l'observation Wie. Cette jeune femme avait une concentration d'inhibiteur de 2,7 u/ml et un taux de facteur VIII de 32 %. Après une grossesse interrompue au 3 mois, suivie de transfusions, l'inhibiteur était monté à 5 1 u/ml et le facteur VIII descendu à 13 %. Transfusions et pénicilline sont administrées si largement que leur rôle étiologique est certainement très sous-estimé.

Les affections qui encadrent la découverte de l'inhibiteur sont assez dissemblables et comparables à celles qui se retrouvent chez les individus possédant un inhibiteur puissant. A noter cependant la maladie de von Willebrand Dans quatre de nos cas existait une atteinte hépatique, une fois au moins sévère.

Il est curieux de trouver chez plus d'un tiers de nos malades, une hérédité de la tendance hémorragique Cependant tous les membres de ces fratries, à l'exception d'un seul, qui ont pu être examinés, ne montrent pas d'inhibiteur Ce n'est donc pas ce dernier qui se transmet, mais un terrain hémorragique mal élucidé.

Clinique (tableaux I IV et V) Dans un peu moins de la moitié des cas, un syndrome hémorragique, jamais menaçant, attire l'attention et amène la découverte de l'inhibiteur Chez un nombre équivalent de sujets souffrant d'une maladie caractérisée un examen systématique de la crase sanguine aboutit, pour des raisons diverses, à la recherche de l'inhibiteur Parfois enfin ce dernier est décelé à la suite d'un contrôle systématique chez un individu présentant une incommodité banale ou bien portant. Le syndrome hémorragique existe chez les $/_2$ des malades pris dans leur ensemble et chez les $3/4$ de ceux dont l'inhibiteur est au dessus de 2 u/ml plus de la moitié présentent un saignement franc. Il n'est d'ailleurs jamais très inquiétant et consiste en épistaxis gingivorragies, ecchymoses, hémorragies génitales, hémorragies des diverses sortes de plaies.

Signes biologiques se rapportant à l'inhibiteur (tableaux II et V) L'hypocoagulabilité est le signe le plus fréquent puisqu'elle se retrouve chez les trois quart du groupe d'ensemble et presque tous les sujets ayant un inhibiteur avéré. Elle se manifeste dans la coagulation du sang

Tableau II Troubles de la crase sanguine en rapport avec l'inhibiteur

Cas N°	Consommation de la prothrombine malade (témoin) sec	TC du PPP sec (N < 210 sec)	du sang, min-sec (N < 12 min)	Facteur VIII % (N = 70–120)	Inhibiteur du facteur VIII u/ml	Facteur IX % (N = 70–120)	Effet inhibiteur du substrat dans le TCT	Particularités
1	28,5 (31 4)	850	12 30	00	6,6	100 inhibiteur 0	+ à 70% de plasma du malade	le PPP un effet inhibiteur à 10% dans le plasma normal
2	normale	normal	17–45	58	5,4		+	
3	normale	370	14 30	13 75	5,1	70 32 inhibiteur 2,7 u/ml	0	2 mois après une grossesse interrompue suivie de transfusions, l'inhibiteur monte de 2,7 à 5,1 u/ml et le facteur VIII tombe de 32 à 13%
4	normale	340	17	70	4,3	85	0	
5	normale	300	12 15	47–61	3,3		0	l'inhibiteur disparaît en même temps que le facteur VIII mont à 61%
6	normale	normal	normal	13	3,3		0	maladie de von WILLEBRAND

Tableau II (suite)

Cas Nº	Consommation de la prothrombine, malade (témoin) sec	TQ du PPP sec (N<240 sec)	du sang, min-sec (N<12 min)	Facteur VIII % (N=70–120)	Inhibiteur du facteur VIII /ml	Facteur IX % (N=70–120)	Effet inhibiteur du substrat dans le TGT	Particularités
7	30,5 (31,5)	275	19–	25–26	3,1	95	0	
8	24,1 (31,5)	normal	15–45	11 100	3,0	85	+ puis 0	l'inhibiteur disparaît en même temps que le facteur VIII monte à 100% et que la consommation se normalise
9	thrombocytopénie	307	normal	60	2,7		0	près adrénaline 0,1 mg disparition de l'inhibiteur et facteur VIII 92%
10	normale	normal	16–45	75	2,0	50–90	+	
11	normale	normal	normal	100	2,0		0	
12	thrombocytopénie	309	normal	100	1,8		substrat déficitaire	
13	normale	normal	12 30	75–100	1 7	35	0	
14	normale	normal	15–15	90	1,6		0	

en dehors des taux des facteurs VIII et IX, seules les valeurs anormales sont portées

Tableau III. Troubles associés de la crase sanguine

Cas Nº	Fragilité vasculaire	Atteintes plaquettaires (plaquettes × 10^9/mm)	Hypoprothrombinémie	Fibrinogène mg/100 ml (N = 300–550)	Fibrinolyse	Facteur X % (N = 70–120)	Anticoagulants circulants ATP: antithromboplastines AT antithrombines
1	0	0	0	278	0	104	0
2	0	0	0	256	0	65	0
3	+	TS 4 min 30 sec a.m. 45 puis normal	0	normal	(+) puis 0	12–90	0
4	(+)	plaquettes 108,0	0	normal	0	88	0
5	0	0	0	normal	0	50	0
6	+	0	0	normal	0	91	0
7	+	0	0	218	0	93	0
8	0	agrégation plaquettaire 0,5 γ/ml 17% (32%) de la valeur initiale	0	normal	0	42–45	0
9	(+)	thrombocytopénie 25,2–60,0	temps facteurs VII et X 17 4 (15,7) sec, temps facteur II 16,1 (15,7) sec	282	0	100	0
10	0	a.m. 36–31	facteurs VII et X 16,8 (15,6) sec facteur II 15,9 (14,8) sec	264	(+)	57 98	présence d une ATP
11	0	plaquettes 316,8	0	766	0	54	présence passagère d'ATP
12	+	plaquettes 57,2	facteurs VII et X 12,4 (15,5) sec facteur II 25,0 (15,0) sec	142	0	11	présence d'une AT
13	(+)	0	facteur V 26,3 (22,2) sec facteurs VII et X 16,4 (15,4) sec facteur II 15,8 (14,9) sec	252	0	77–85	présence d'une ATP
14	0	0	0	normal	0	93	0

Tableau IV Circonstances étiologiques

Sexe	Hommes : 5 Femmes : 9
Age	< 20 ans : 9 20–39 ans : 2 40–59 ans : 3
Tendance hémorragique dans la fratrie	5 1 présence d'un inhibiteur faible du facteur VIII
Grossesses	4
Transfusions	7
Pénicilline	1
Mise en évidence de l'inhibiteur	par contrôle systématique 2 par contrôle d'un syndrome hémorragique 6 épistaxis (1), hémarthrose (1), métrorragies (2) tendance hémorragique (2) par contrôle au cours d'une affection caractérisée 6 splénomégalies chroniques (2) abcès sous-phrénique sur nécrose hépatique post-traumatique réséquée (1) [illegible] avec hépatite d'inoculation (1) syndrome fébrile avec arthralgies et crises abdominales (1) maladie de von Willebrand (1)

complet et un peu moins dans celle du plasma pauvre en plaquettes après recalcification.

La consommation de la prothrombine est mauvaise, une fois éliminée toute thrombocytopénie dans un peu moins d'un tiers des inhibiteurs avérés. Quoique inconstant, c'est là un signe intéressant, car en l'absence de toute autre cause, il attire l'attention sur l'inhibiteur. Les relations entre la consommation de la prothrombine et l'inhibiteur ainsi que l'effet inhibiteur du plasma dans le TGT sont illustrés par l'observation N. 3.

Le facteur VIII est le plus souvent abaissé pour l'ensemble des cas; il l'est presque toujours chez les sujets à inhibiteur avéré. C'est là aussi un signe remarquable, qui fait soupçonner un inhibiteur du facteur VIII, cependant inconstant: l'observation N. 1 qui présente la plus forte concentration d'inhibiteur de notre série (6,6 u/ml) a un taux normal de facteur VIII (80%). Cependant le lien entre le facteur VIII et l'inhibiteur est indéniable: l'un s'abaisse en même temps que l'autre s'accroît et inversement (cas N. 3, 5, 8).

La mise en évidence d'un effet inhibiteur, en dehors du test d'incubation spécifique, est difficile avec un matériel aussi peu actif. Le

Tableau V Manifestations cliniques et biologiques de l'inhibiteur

	Total des cas	Cas à inhibiteur > 2 u/ml	Cas douteux (inhibiteur < 2 u/ml)
Syndrome hémorragique :	10 : 14 cas	7 : 9 cas	3 : 5 cas
franc	6 : 14 cas	5 : 9 cas	1 : 5 cas
discret	4 : 14 cas	2 : 9 cas	2 : 5 cas
Hypocoagulabilité	11 : 14 cas	8 : 9 cas	3 : 5 cas
du plasma pauvre en plaquettes	7 : 14 cas	6 : 9 cas	1 : 5 cas
du sang complet	10 : 14 cas	7 : 9 cas	3 : 5 cas
Mauvaise consommation de la prothrombine	3 : 12 cas[1]	3 : 8 cas	0 : 5 cas
Facteur VIII abaissé (< 70%)	8 : 14 cas	8 : 9 cas	0 : 5 cas
taux moyen, % (N = 90 ± 26)	58 ± 30	42 ± 27	88 ± 13
Effet inhibiteur du plasma dans le TGT	4 : 13[2]	3 : 9 cas	1 : 4 cas[2]

[1] Deux cas avec thrombocytopénie et mauvaise consommation de la prothrombine ont été éliminés.

[2] Un cas avec substrat déficitaire au temps de Quick éliminé.

plasma pauvre en plaquettes utilisé comme substrat d'une mixture normale dans le TGT ne s'est montré inhibiteur que dans un tiers de cas. Son temps de recalcification est rarement assez allongé pour permettre une recherche d'anticoagulant par mélange gradué à un plasma normal. Seule l'observation N° 1 avec ses 6,6 u/ml d'inhibiteur a permis cette épreuve, montrant un effet inhibiteur déjà manifeste au $^1/_{16}$ ainsi qu'une autre du même ordre avec le mélange des substrats dans le TGT positive au $^1/_8$.

Le facteur IX est abaissé dans un tiers des cas où il a été testé. Ce qui retient surtout l'attention est, dans l'observation N° 3, la coexistance d'un inhibiteur du facteur IX avec celui du facteur VIII et à un taux identique (2,7 u/ml) en même temps que le facteur IX était déprimé à 32%. Au contraire, l'observation N° 1 avec 100% de facteur IX, ne montre pas trace d'inhibiteur anti facteur IX.

Signes biologiques associés à ceux de l'inhibiteur (tableaux III et VI) Certains affectent également les cas douteux et ceux à inhibiteur avéré: fragilité vasculaire, abaissement du fibrinogène, fibrinolyses. D'autres prédominent dans les cas douteux: hypoprothrombinémies,

Tableau VI. Troubles associés de la crase sanguine

	Total des cas	Cas à inhibiteur > 2 u/ml	Cas douteux (inhibiteur < 2 u/ml)
Fragilité vasculaire	7 : 14 cas	5 9 cas	2 5 cas
Hypoprothrombinémies	4 14 cas	1 : 9 cas	3 5 cas
Atteintes plaquettaires	7 : 14 cas	4 9 cas	3 5 cas
Thrombocytopénies	3 14 cas	1 : 9 cas	2 5 cas
Atteintes fonctionnelles	3 : 14 cas	2 9 cas	1 : 5 cas
Abaissement du fibrinogène	7 14 cas (dont 1 hépatite grave)	4 : 9 cas	3 5 cas (dont 1 hépatite grave)
Fibrinolyses (larvées) passagères	2 : 14 cas	1 9 cas	1 5 cas
Antithromboplastines	3 14 cas	0 : 9 cas	3 : 5 cas
Facteur IX < 70%	3 7 cas		
Facteur X < 70%	6 : 14 cas	3 9 cas	3 4 cas

Dans 3 cas, atteinte hépatique dont 1 hépatite grave avec hypoproaccélérinémie, hypofibrinogénémie et présence d'une antithrombine.

thrombocytopénies, faible activité antithromboplastique du plasma. Les uns et les autres paraissent sans lien avec l'inhibiteur Les atteintes fonctionnelles des plaquettes pourraient soulever la question d'un rapport de causalité, n était leur nombre infime.

Caractéristiques évolutives L'inhibiteur n est pas constant. Nous le voyons augmenter après une grossesse suivie de transfusions (cas N 3) Chez d autres sujets, il disparait, en même temps que certains signes s évanouissent et que d autres persistent.

Cas N° 8 TC 15 min 30 sec. Consommation de la prothrombine 24,1 (31,5) sec. Effet inhibiteur du plasma sur une mixture normale dans le TGT Facteur VIII 11%. Inhibiteur 3 u/ml. Après 10 jours, TC 15 min 45 sec. Consommation normale. Disparition de l'effet inhibiteur dans le TGT Facteur VIII 100%, plus d'inhibiteur

Cas N° 5 TC 12 min 15 sec. Plasma pauvre en plaquettes recalcifié 360 sec. Facteur VIII 47%. Inhibiteur 3,3 u/ml. Après deux mois, TC et plasma pauvre en plaquettes recalcifié normaux. Facteur VIII 64%. Inhibiteur non appréciable.

La disparition de l'inhibiteur après une injection intraveineuse de 0 08 mg d adrénaline dans un cas d'hépatosplénomégalie résulte sans doute de sa neutralisation par l excès de facteur VIII libéré à la suite de cette épreuve.

Caractéristiques biochimiques Nos cas ne se prêtaient guère à une analyse biochimique. Tous nos inhibiteurs ont résisté à une double

absorption par le sulfate de baryum. Le sérum du cas N° 1 était presque aussi actif que le plasma baryté (5,5 u/ml) Nous avons essayé de fractionner le plasma baryté du cas N 3 par électrophorèse sur cellogel (méthode modifiée par expression des blocs à la place de l élution) Nous avons retrouvé 5 7 u/ml d inhibiteur dans les α-globulines et 2,4 u/ml dans les β-globulines.

Discussion

La littérature des inhibiteurs du facteur VIII est devenue considérable [17 26] depuis que LOZNER *et al.* [16] en publièrent le premier cas spontané et que MUNRO et JONES [19] les trouvèrent chez l'hémophile classique A. Aujourd'hui, la plupart des auteurs les considèrent comme des anticorps (29 14) 7 S γ-globulines [1 28] associés à des 19 S γ-M globulines [30] Ig globulines à chaines légères, semblables aux autoanticorps froids des anémies hémolytiques [27] Cependant tous ne sont peut-être pas des γ-globulines [24 25] De plus, des substances très différentes telles que l hématoporphyrine, peuvent se comporter comme des inhibiteurs caractéristiques du facteur VIII [8] Il n est d ailleurs pas certain que tous ces inhibiteurs soient des anti corps au moins dans certains cas, ils fonctionnent comme des enzymes [6 22] Les inhibiteurs du facteur VIII spontanés et ceux apparus chez des hémophiles sont considérés comme identiques et étudiés ensemble. Mais celà même n est pas assuré puisque leur cinétique peut se montrer différente [12] Ces divergences doivent nous faire réserver notre jugement sur l unicité de ce groupe d inhibiteurs.

Les conditions étiologiques qui entourent la production des inhibiteurs spontanés sont assez bigarrées. Cependant la grossesse [5 9 16b, 20] même chez une primipare la sensibilisation à la pénicilline [12] jouent un rôle manifeste. Tout un ensemble de malades est rassemblé sous l appellation plutôt confuse de maladies immunologiques. Un groupe assez notable en est formé par divers rhumatismes, puis viennent les atteintes lymphoïdes, les dysprotéinémies, des affections cutanées intestinales, hépatospléniques. Pour le reste, dans beaucoup d observations, aucune étiologie ne se laisse déceler Chez tous ces malades, la corticothérapie n a guère donné de succès [13] et pourrait même être suspectée dans la genèse de l inhibiteur [11] Les immunosuppresseurs sont à l essai [32]

Nous croyons que les inhibiteurs faibles du facteur VIII que nous avons détectés sont de même nature que ceux, puissants et spectaculaires, dont parle la littérature. Nous trouvons ici le même cadre étiologique, la grossesse surtout et les transfusions, suggérant une immunisation. Par ailleurs, nous avons recherché aussi cet inhibiteur faible chez les hémophiles. Nous l'avons trouvé (> 2 u/ml) chez plus d'un tiers de nos hémophiles graves (5 16) qui avaient reçu de nombreuses transfusions, mais chez aucun de nos hémophiles de la variété mitis, peu transfusés (0 8) L identité entre l'inhibiteur faible et le fort est donc vraisemblable, encore que le groupe d ensemble puisse réunir des cas de pathogénie et de signification fort diverses.

Quelques remarques s'imposent cependant l association d un inhibiteur du facteur VIII avec un inhibiteur du facteur IX, comme chez le cas N 3 a été signalée [25] L'hérédité d une tendance hémorragique, que nous relevons assez souvent parmi nos malades, n est guère mentionnée que par quelques auteurs [21 33] Nous sommes frappés par le jeune âge de notre population alors que l inhibiteur classique touche souvent des sujets plus âgés et même des vieillards. Si ce fait se vérifiait, il pourrait signifier que, contrairement aux apparences, les inhibiteurs puissants ne naissent pas toujours ex abrupto mais qu un certain nombre d'entre eux se développent à partir d un état latent, muet ou discret en clinique, caractérisé par un faible taux d inhibiteur et une médiocre tendance hémorragique Chez les hémophiles, nous l'avons constaté, cette situation s accompagne encore d une très bonne réponse aux transfusions de sang et de plasma qui corrigent bien le temps de coagulation et même la consommation de la prothrombine l'inhibiteur disparaît ou diminue passagèrement, pour réapparaître et remonter ensuite Ce mauvais équilibre, gros de dangers futurs, peut brusquement, sous une stimulation énergique ou maintenue, antigénique ou autre, s effondrer

Si donc ce faible inhibiteur du facteur VIII n a pas de grand rôle à jouer en clinique et s'il ne rend même pas son porteur vraiment malade, le mettre en évidence n est pourtant pas inutile. Car les sujets qui en sont affligés, sont à surveiller de près. Les grossesses, les transfusions et la pénicilline dont on abuse, d'autres facteurs encore que nous ignorons risquent d accroître cet inhibiteur et nous devons craindre, sans néanmoins pouvoir le prouver que parmi ces sujets, puisse naître un inhibiteur puissant ou pour le moins malfaisant.

Résumé

Certains sujets présentent un inhibiteur faible du facteur VIII, manifesté par une petite hypocoagulabilité, parfois par une mauvaise consommation de la prothrombine, et surtout un taux réduit du facteur VIII. Les grossesses, les transfusions, la pénicilline, une tendance héréditaire aux hémorragies jouent un rôle étiologique. Ces inhibiteurs faibles sont probablement de même nature que les grands inhibiteurs de la littérature et représentent un état presque latent mais qui pourrait se transformer brusquement un jour en inhibiteur puissant. D'où la nécessité chez de tels sujets de surveiller les grossesses et d'économiser transfusions et pénicilline.

Summary

Some individuals possess weak factor VIII inhibitor activity as shown by minor hypocoagulability sometimes by poor prothrombin consumption and especially by low factor VIII levels. Pregnancy transfusions, penicillin, hereditary haemorrhagic tendency all play a part in the aetiology These inhibitors represent an almost latent state, which could, however t some point be transformed into powerful inhibitor This makes it necessary in such patients for close supervision during pregnancy and caution in the use of transfusions and penicillin.

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Adresse des auteurs: Prof. Dr Georges Mayer, Dr Geneviève Noel et Prof. Dr R. Waitz, Centre de Transfusion Sanguine, 10, rue Spielmann, 67 Strasbourg (France).

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Department of Anatomy University of Bristol, and University Department of Haematology Royal Infirmary Sheffield

Cell Degeneration Pattern in Bone Marrow and Blood of Guinea Pigs Following Administration of Cyclophosphamide

M. Kaul[1] and G. Hudson

In a quantitative study of haemopoietic recovery over a period of 6–21 days following the injection of a sublethal dose of cyclophosphamide in the guinea pig [8] striking differences were found in the recovery pattern of different cell-lines in the bone marrow These differences could be attributed to a complex interplay of factors, and in relation to this it seemed important to have more complete information about the pattern of degeneration following the use of this chemotherapeutic agent. In the investigation described here, quantitative cellular studies, supplemented by light and electron microscopic observations, have been made on guinea pigs at intervals of 6–72 h after the injection of a similar sublethal dose of cyclophosphamide. Some of the findings have been mentioned in a brief abstract elsewhere [7]

Material and Methods

All the observations were made on healthy male guinea pigs of the Dunkin-Hartley strain weighing approximately 400 g at the commencement of the experiment. A total of 42 animals was used, 21 in the test and 21 in the control series. Each animal in the test series was given a single i.p. injection of 350 mg/kg cyclophosphamide, and each control was injected with a corresponding volume of sterile physiological saline. Pilot experiments in which animals were observed over a period of 3 months, have shown that this is not a lethal dose of cyclophosphamide. Over the period of 3 days studied here, the animals were a little off-colour and showed a loss of body weight of about 10%. No intestinal disturbances were observed. Quantitative cellular studies of blood and bone marrow were made in 5 animals

[1] Nuffield Research Fellow

per group at 6, 24 and 72 h. Specimens of marrow were examined with the light and electron microscope in two animals per group after the same time intervals.

The techniques used for quantitative study of blood and bone marrow were, except as mentioned below, identical with those used in the earlier study [6]. The animals were killed by exsanguination from the common carotid artery under ether anaesthesia. Routine counts were performed on the arterial blood, a direct method of counting eosinophils [5] being employed. Using procedures described in detail by YOFFEY [13], a marrow suspension of known dilution was prepared, by shaking a plug of marrow with autologous serum in a small tube containing a glass bead. Normal haemocytometric methods were used to count the number of nucleated cells in the suspension and the result was converted into total nucleated cells per unit volume of bone marrow. The procedure was carried out on humeral marrow of both sides and the results were averaged. Dry smears were prepared from the suspensions and stained with MACNEAL tetrachrome stain. Differential counts were made at right angles to the long axis of the smear at approximately its centre. By relating the percentages of the various cell types to the total nucleated cell counts, the results could finally be expressed as absolute numbers per mm³ of the original marrow.

With our usual techniques, one can normally expect that between 5 and 15% of all the cells seen in the marrow smears will be damaged to such a degree that accurate identification is impossible. The proportion of such damaged forms tends to be less in thicker smears. Preliminary observations on marrow smears at 24 and 72 h after cyclophosphamide indicated that a much higher proportion of damaged forms – of the order of 40% – was present. It seemed possible that this difference might be related to the fact that the marrow was relatively acellular (see below) and that the concentration of cells in the suspension was correspondingly low. When more concentrated suspensions of marrow in serum, 1 in 5 or 1 in 6, instead of the usual 1 in 10 or 1 in 12, were used, the proportion of damaged forms was considerably less and usually lay within normal limits. Concentrations of this order were, therefore, employed in the main series of experiments described above.

The techniques used for obtaining specimens of bone marrow for light and electron microscopic study were essentially similar to those described previously [6]. Within 4 min of the demise of the animal, specimens of humeral marrow were placed in ice-cold 1% osmium tetroxide buffered at pH 7.3 and cut up into very small blocks. These were fixed for 1.5 h, washed, dehydrated through increasing strengths of alcohol and finally embedded in Araldite. Sections of about 1 μm thickness were stained with aqueous solutions of methylene blue and azur II in borax and examined with the light microscope. Ultrathin sections were stained with either 1% uranyl acetate or 1% phosphotungstic acid for 5 min and examined in a Hitachi HS 7 electron microscope.

Results

The quantitative data are summarized in table I and the principal findings seem self-evident. It should be noted that the absolute counts of some subgroups in the bone marrow and the blood (including basophil granulocytes, monocytes, plasmacytes, megakaryocytes, macrophages and unidentified cells) have not been separately itemized. In the case of very large cells such as megakaryocytes and macrophages, which show uneven distribution in marrow smears, the quantitative technique does not provide reliable estimates of their numbers.

Table I Quantitative data

	6 h		24 h		72 h	
	Test	Control	Test	Control	Test	Control
Marrow cells × 1000/mm³						
Total nucleated	1 799 (98)	1,804 (83)	746 (136)	1,846 (60)	454 (80)	1,878 (69)
Total myeloid	615 (151)	687 (143)	434 (131)	561 (163)	244 (77)	757 (95)
Myeloblasts	13 (8)	19 (8)	4 (3)	28 (8)	3 (2)	25 (7)
N. promyel + myel.	54 (33)	66 (39)	14 (3)	58 (16)	5 (4)	78 (22)
N. metamyel.	175 (46)	108 (44)	77 (27)	102 (36)	36 (10)	134 (45)
N band + segm.	273 (111)	376 (105)	232 (123)	295 (114)	121 (58)	405 (86)
N unidentified	41 (3)	50 (26)	38 (31)	22 (6)	36 (15)	52 (33)
Eosinophils	41 (14)	61 (15)	40 (16)	47 (23)	38 (15)	32 (19)
Total erythroid	448 (157)	481 (138)	70 (34)	609 (156)	46 (37)	507 (44)
Proerythrobl.	14 (5)	24 (12)	1 (1)	16 (9)	1 (1)	18 (10)
Basophilic	30 (23)	55 (10)	1 (1)	50 (28)	2 (2)	54 (18)
Poly + ortho.	381 (138)	389 (133)	62 (32)	535 (124)	40 (34)	427 (56)
Total lymphoid.	*469 (168)*	*349 (46)*	*151 (79)*	*396 (74)*	*59 (25)*	*327 (137)*
Small lymphoc.	386 (146)	220 (35)	134 (70)	309 (75)	45 (21)	245 (142)
Transitional	32 (13)	55 (15)	13 (12)	54 (15)	8 (6)	32 (11)
Total damaged	172 (29)	147 (31)	63 (22)	151 (50)	67 (15)	119 (42)
Blood cells 100/mm³						
Total white	87 (25)	68 (15)	37 (9)	55 (10)	13 (6)	61 (8)
Total neutro.	61 (23)	36 (16)	28 (5)	23 (2)	4 (2)	31 (7)
Eosinophils	0.15 (0.11)	0.38 (0.18)	0.04 (0.04)	0.38 (0.06)	0.06 (0.02)	[1]0.28 (0.13)
Total agranular	25 (6)	28 (3)	7 (3)	25 (9)	8 (6)	27 (6)
Spleen weight mg	570 (70)	570 (190)	400 (70)	670 (120)	380 (70)	740 (80)

Average values are given with standard deviations in brackets.
Difference between test and control values significant at the 0.01 level of probability

In the marrow smears, as well as in the electron microscopic preparations obtained from animals at 24 and 72 h after cyclophosphamide neutrophil granulocytes were encountered with certain abnormal features (fig 1 2) Typically these cells had the size and cytoplasmic characteristics of immature neutrophil leucocytes while the nucleus showed marked segmentation The number of separate nuclear segments often exceeded 10. For purposes of classification we termed such cells segmented metamyelocytes The average counts of these forms were 41 000 (±22 000) at 24 h and 33 000 (±10 000) at 72 h A few



Fig 1 'Segmented metamyelocyte in bone marrow smear 24 h after cyclophosphamide. The nucleus shows an advanced degree of segmentation nine lobes could be identified. The cell is large, being about 17 μm in diameter The cytoplasm shows numerous (neutrophilic) granules as well as dense inclusion. I the original smear the cytoplasm appeared slightly basophilic (2,800)

segmented metamyelocytes were seen in 3 of the experiments at 6 h after cyclophosphamide. One also gained the impression that at 24 and 72 h, the later forms of neutrophil showed an increased degree of nuclear segmentation but this was not studied quantitatively

In the blood smears, the neutrophil granulocytes of the 24 and 72 h test animals showed clear evidence of increased segmentation. Arneth counts carried out on the 24 h specimens indicated that there was a marked shift to the right' in the cyclophosphamide injected group. In particular there was a highly significant increase in the percentage of neutrophils with 5 or more nuclear segments (51% as against 11 % in the controls t=4 1) Nuclei with up to 14 segments were noted. Some cells also showed cytoplasmic basophilia and were reminiscent of the segmented metamyelocytes observed in the marrow

In sections examined with both the light and electron microscope, the bone marrow of the test animals showed little difference from that of the controls at 6 h, although macrophages containing degenerating cells seemed more frequent. At 24 h, the test marrows appeared rather acellular and macrophages containing the remains of degenerating cells were particularly prominent. At 72 h the test marrows were again

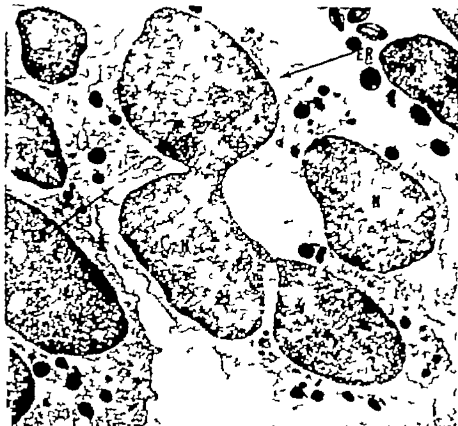


Fig. 2. Electron micrograph of segmented metamyelocyte' 24 h after cyclophosphamide. The nuclear profiles (NN) show evidence of segmentation. The cytoplasm contains well-developed endoplasmic reticulum (ER) as well as a number of dense (neutrophilic) granules (×7,900).

less cellular than those of the controls fat vacuoles and large sinusoids appeared more numerous.

Discussion

The results show that following the administration of a sublethal dose of cyclophosphamide, there was a profound decrease in marrow cellularity No significant change was observed in the total number of nucleated cells at 6 h, but at 24 and 72 h, striking changes were present. The three major cell-groups seemed to be affected somewhat differently A very considerable reduction in the numbers of erythroid and lymphocytic cells was already present at 24 h, whereas no signif

icant change was detected in the total number of myeloid cells at this interval. At 72 h, the average numbers of erythroid, lymphocytic and myeloid cells were reduced respectively to 9 18 and 32 of the corresponding control values. A similar sensitivity' of erythroid precursor cells and lymphocyte-like cells to cyclophosphamide has been observed by HOST [4] in the rat. In this connection it may also be relevant that by 24 h there was a significant reduction both in the counts of the mononuclear cells (lymphocytes, monocytes and blast cells) of the blood as well as in the weight of the spleen, whereas no significant change in blood neutrophil numbers was detected until 72 h. To what extent an early reduction in the size of a particular cell population reflected an actual destruction of cells is uncertain, although the prominence in the 24 h sections of macrophages containing degenerating cells would support such a concept. The suggestion that the myeloid series may have been less affected by cyclophosphamide in this respect, might also be correlated with the observation that during recovery from this drug [8] total myeloid numbers regained (and in fact overshot) control levels approximately a week before the erythroid and lymphocyte numbers did. However attention must also be given to the rather complex changes in the myeloid sub-groups. It may be seen from table I that over half the normal myeloid cell-population consists of band and segmented neutrophils, and that at 24 h after cyclophosphamide the number of these later forms was virtually unaffected. In contrast, the early actively-dividing forms (myeloblasts, promyelocytes and myelocytes) showed a dramatic reduction in numbers at 24 h. By 72 h, the counts of the later forms of marrow neutrophil as well as the counts of neutrophils in the blood, showed a highly significant decrease by 144 h [8] they had reached levels of less than 5% of the corresponding controls. These observations appear to be consistent with those of HOST [4] who found that in the rat, the non-dividing myeloid cells reached minimum values two days later than the dividing myeloid cells, and with those of HARRIS [3] who reported that in guinea pigs following exposure to irradiation, the decline in the number of granulocytes in the early stages varied inversely with their degree of differentiation. The present finding that total myeloid numbers were not greatly reduced at 24 h after cyclophosphamide appears to have been due almost exclusively to a resistance of the later forms of neutrophil. It would also seem that the eventual reduction in the size of the marrow compartment of band and segmented neutrophils

reflected the fact that the earlier forms had either been destroyed or were failing to mature. Thus, very few new cells would enter the compartment of later forms to replace those being released into the blood stream.

The presence in the bone marrow of the cells which we have designated 'segmented metamyelocytes' (fig 1 2) may be very relevant to this interpretation. In smears, these usually appeared as large cells with basophilic cytoplasm containing neutrophil granules, and with a nucleus which showed a number of separate segments. It is of course, true that in normal marrow smears, one may infrequently encounter an immature neutrophil with two or more distinct nuclear segments. Studies in tissue culture have also shown that in maturing granulocytes, segmentation appears and disappears several times before it becomes a permanent morphological feature [1] However the frequency with which segmented metamyelocytes were observed following cyclophosphamide and the degree of segmentation which was present in some of these cells seemed clearly to indicate that they were abnormal Confirmation of this was found in a preliminary experiment which we carried out in collaboration with Dr E. S MEEK. Measurements with an integrating microdensitometer indicated that segmented metamyelocytes contained double the normal amount of nuclear DNA. Large cells with abnormally large amounts of DNA (in most instances not exceeding the pre mitotic content) have been noted by a number of other investigators after the use of cytotoxic drugs, and the suggestion has been made that progression is blocked at the G2 stage of the cell-cycle (see the reviews of KILLMAN [9] and WHEELER [12]) Whatever the precise mechanism by which segmented metamyelocytes were produced, it would eventually lead to a decreased production of the mature forms of neutrophil granulocyte.

The presence of increased nuclear segmentation in neutrophil granulocytes, which was seen here both in the blood and in the bone marrow has been reported by other investigators following the use of alkylating agents [2 10, 11] The evidence of increased nuclear segmentation in the blood neutrophils at 24 h after cyclophosphamide suggests that this agent may have an effect on non-dividing as well as on dividing cells. The morphological appearances resemble those of a premature and rather exaggerated ageing process. The possibility that these appearances may parallel changes in cell-function might repay further investigation

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Summary

Studies of bone marrow and blood were carried out following a single sublethal dose of cyclophosphamide in guinea pigs. At 24 h, there was a profound decrease in marrow cellularity and macrophages containing degenerating cells were prominent. Erythroid, lymphocytic and immature myeloid cells were reduced in numbers, while later forms of neutrophil granulocytes were virtually unaffected. There was reduction in the spleen weight and in the number of mononuclear cells in the blood. At 72 h later forms of marrow neutrophil and blood neutrophils were also significantly reduced.

The presence in the bone marrow of abnormal neutrophils, designated as 'segmented metamyelocytes' and nuclear hypersegmentation in the neutrophils of the blood seem particularly significant in relation to the mechanism of action of cyclophosphamide.

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Authors' addresses: Dr. G. Hudson, Faculty of Medicine, University of Sheffield, *Sheffield, S10 2TA* (England); Dr. M. Kaul, Department of Anatomy, Lady Hardinge Medical College, *New Delhi* (India)

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Departments of Medicine and Pathology, Veterans Administration Hospital
and School of Medicine, University of Pittsburgh, Pittsburgh, Pa.

Histiocytic Medullary Reticulosis

Case Report with Electron Microscopic Study[1]

Z. A. Zawadzki, C. E. Peña and E. R. Fisher

In 1939 Scott and Robb-Smith [1] described the clinical and pathological features of 4 patients which they considered to be unique from other forms of malignant lymphoma. The lesion which they designated as histiocytic medullary reticulosis was characterized by focal proliferations of histiocytic cells in medullary portions of hemopoietic organs. Erythrophagocytosis by the histiocytic cells represented a distinctive feature of the disorder. This, as well as the absence of *involvement of the peripheral blood, distinguished histiocytic medullary* reticulosis from monocytic and histiocytic leukemia.

Histiocytic medullary reticulosis is a rapidly progressive fatal disorder [2–16] with survival usually from only 1 to 15 months with a median duration of 5½ months following recognition [5–10]. No sex predilection has been noted and the age of patients with the disorder has ranged from 26 to 70 years with a mean of 48 years [5].

Recently we had the opportunity to study a patient in whom *intra vitam* diagnosis of this disorder was established. Electron microscopy of samples of bone marrow has allowed for the ultrastructural characterization of these neoplastic elements.

Case Report

A 45 year old white man entered the hospital complaining of intermittent pains in both gluteal and lumbar regions radiating to the medial aspects of both thighs. He also had experienced general malaise, anorexia and constipation for several months. He had lost

[1] This investigation was supported by United States Public Health Service grant C-5195.

10 kg since the onset of his illness. His past medical history had disclosed that he was subjected to two craniotomies for brain tumor 6 years previously and had received cobalt therapy for this lesion 1 year ago. The liver edge was tender and palpable 3 cm below the right costal margin. The tip of the spleen was felt just below the left costal margin. Discrete small (1 × 1 cm) axillary lymph nodes were palpable bilaterally. There was a striking bony tenderness over the sternum, rib cage, both clavicles, both scapulae, pelvis and the spinous processes of the vertebrae. Neurological examination was negative although symptoms of expressive aphasia were present. Hematocrit 32%, hemoglobin 10.8g%. White cells 6,600/mm³ with 66% neutrophils, 1% myelocytes, 26% lymphocytes, 5% monocytes, 1% eosinophils and 1% basophils. Peripheral blood smear showed polychromatophilia, red cell stippling, spherocytes and a few nucleated red cells. Reticulocytes varied from 1.0 to 2.8%. Platelets 154,000/mm³. Sedimentation rate 72 mm/h (corrected to 18 mm/h). Total serum protein 6.6 g% (albumin 2.9 and globulin 3.7). Serum bilirubin 0.7 mg%, cholesterol 230 mg% and alkaline phosphatase 32 King-Armstrong units. Glutamic oxaloacetic transaminase (SGOT) 45 units, lactic dehydrogenase (LDH) 920 units. LE test, Coombs test and the serological examination for syphilis and rheumatoid factor were negative. The leukocyte alkaline phosphatase score was 76 (normal range 20–100). Serum electrophoretic pattern: moderate elevation of α_2-globulins. Serum immuno-electrophoretic patterns were within normal limits.

Radiological skeletal survey showed an increased density of bones of the vertebral bodies and the pelvis in both sacroiliac regions. Aspiration of bone marrow was difficult due to the hardness of bone. Biopsies of bone marrow were performed.

Bone Marrow

Light microscopy. The bone marrow smear was hypercellular and consisted almost exclusively of large reticulum cells whose average size was 30–40 μm. Most of these cells contained 2 or 3 nuclei and a few nucleoli. Almost every cell contained one or more erythrocytes. Occasionally erythroblasts could be recognized within the cytoplasm of these large reticulum cells. Smears stained with Prussian blue revealed a moderately positive reaction for iron within the reticulum cells but an absence of extracellular iron stores.

A liver biopsy disclosed foci of extramedullary hematopoiesis and marked hemosiderosis.

Electron microscopy. The cells comprising the lesion of histiocytic medullary reticulosis exhibited large nuclei which were frequently indented. Nuclear chromatin was evenly dispersed. Nucleoli were inconspicuous. The nucleus was surrounded by a double membrane. Cell cytoplasm was voluminous and its surface was often irregular with pseudopodal extensions. Varying sized pinocytic vesicles were also present beneath the cell membrane. Golgi structures appeared well developed and were observed in perinuclear areas. Coarse endoplasmic reticulum frequently appeared as relatively large, occasionally tortuous channels (fig. 1). Other vesicles and vacuoles were dispersed throughout the cytoplasm. Occasional dense bodies surrounded by a solitary membrane with a homogeneous matrix (cytosomes) or portions of other organelles within their confines (cytosegrosomes) were also present (fig. 2). Tetrads characteristic of ferritin could be discerned in some of the membrane-bound bodies. Mitochondria were relatively sparse and characteristically contained only a modicum of cristae. The cytoplasm of some of these cells contained numerous homogeneous or multivesiculated lipid droplets and/or myelin figures whereas others contained fine fibrils. Portions of intact forms of erythrocytes were encountered within the cytoplasm of some cells (fig. 3). In some instances, erythrocytes were encountered between the cell membranes of adjacent tumor cells. In such instances, they appeared distorted (fig. 4). Other erythrocytes appeared to indent the cell membrane of tumor cells and portions of phagocytized erythrocytes at the cell surface were often associated with large cytoplasmic vacuoles. A solitary membrane frequently was noted about intracytoplasmic portions of such erythrocytes.

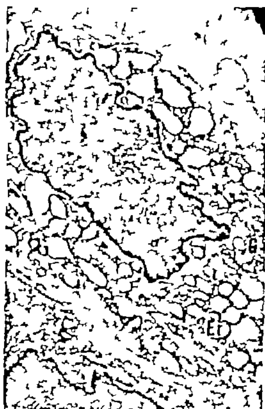


Fig 1 Portions of two neoplastic cells in bone marrow Endoplasmic reticulum (*Er*) is dilated and Golgi complex (G) is conspicuous. The nucleus is scalloped (× 12,000)

Erythrokinetic Studies

The serum iron was 190 μg/100 mL. Total iron binding capacity was 233 μg/100 mL. The red cell survival half-time, determined by chromium[51] method was 28.5 days (normal range 25 to 37 days) Plasma clearance of radioacti iron was 67 min (normal range 60–90 min) Direct counts over spleen, liver heart and sacrum revealed progressi accumulation of radioactivity in the spleen, minor rise in the liver the usual drop in the heart and essentially no uptake over the sacrum. No essential difference in the radioactivity uptake over the sacrum in comparison to the spleen and the liver was observed during the duration of study Blood counts during this time showed plate level from the 5th day on. The plateau of the curve and low level of radioactivity indicated poor incorporation of radioiron.

Clinical Course

The patient was observed in the outpatient clinic monthly intervals. The hematocrit ranged from 19 to 27 and the hemoglobin from 8.4 to 6.0 g%. H was readmitted to the hospital 7 months after the onset of his illness because of mass hematemesis from which he succumbed.

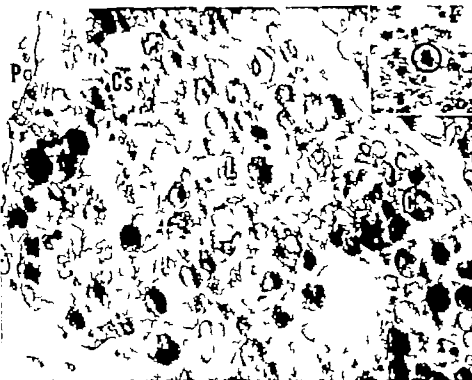


Fig. 2. Portion of neoplastic cell in bone marrow with cytoplasmic lipid droplets (L). Cytosegresomes (Cs) and cytosomes (C) are also present. The cytoplasmic border shows pseudopodal extensions (P) (9,900). The insert reveals tetrads of ferritin in the cytosome indicated at C (350,000).

Autopsy

Macroscopie description. The liver weighed 2,700 g. The parenchyma appeared mottled yellow-brown. The spleen weighed 300 g and was soft with dark red cut surface. The para-aortic and cervical lymph nodes were moderately enlarged; the cut surface was white and homogeneous. Tumor nodules were in the left temporo-occipital area, frontal horns of both lateral ventricles and occluding the fourth ventricle of the brain.

Microscopic description. The liver and spleen contained numerous foci of hematopoiesis. Kupffer cells of the former contained intact red blood cells as well as hemosiderin within their cytoplasm. The bone marrow and lymph nodes revealed almost total replacement of their normal structure by sheets of histiocytes (fig. 3). These latter had abundant finely granular cytoplasm that was faintly reactive when stained by the periodic acid-Schiff technique and often contained oil red O positive lipid droplets and erythrocytes. Cell nuclei were for the most part eccentrically placed, ovoid and reniform and with finely granular chromatin. Focal infiltrates of these cells were also seen in the pancreas and bone marrow.



Fig. 3. Erythrocyte (E) within cytoplasm of neoplastic cell. There are relatively few organelles in this area of the cytoplasm (16,500).

Sections of tumor nodules in the brain showed a moderately well differentiated ependymoma with large areas of oligodendroglial and astrocytic differentiation as well as multiple ependymal neoplasms.

Discussion

The clinical and pathological features encountered in this patient warrant the diagnosis of histiocytic medullary reticulosis [1, 5, 6, 10, 11, 16]. The etiology of this disorder has not been established [5, 6]. There is little evidence to implicate bacterial infection or autoimmunity in its pathogenesis, as has been suggested [2, 6]. On the other hand, most have agreed that it represents a neoplasm of histiocytic elements. Its distinction from so-called histiocytic leukemia or reticulum cell sarcoma is apparent since a leukemic phase of the disease has not



Fig 4 Distorted erythrocyte (E) between the cytoplasmic membranes of two adjacent neoplastic cells. A large vacuole is present in the cytoplasm of one of the cells and the erythrocyte markedly invaginates the cytoplasm of the other (21 700)

been recognized and erythrophagocytosis is not recognized as a salient feature of these two diseases. The electron microscopic appearance of the tumor cells allows for their categorization as histiocytes, not only because of their overt phagocytic properties but also the presence of pseudopodal extensions, frequent intracytoplasmic cytosomes and cytosegresomes and quantity and appearance of other subcellular organelles. The sequential changes occurring in erythrophagocytosis as revealed by electron microscopy does not appear unique in this instance the findings do not explain why these histiocytic elements have such an avidity for erythrocytes. We have observed qualitatively similar erythrophagocytosis by electron microscopy in a few examples of ZIEVE's syndrome and other hemolytic anemias.



Fig. 5. Neoplastic cells replacing bone marrow in section of *post mortem* specimen stained by WILDER's reticulum method (×100)

The results of the ferrokinetic studies as well as the presence of accumulations of ferritin within the cytoplasm of the tumor cells are consonant with the view that the anemia in patients with histiocytic medullary reticulosis may be due to the lack of iron stores as result of the excessive erythrophagocytosis. Estimates of erythrocyte survival time and iron clearance were within normal limits

The cerebral neoplasm encountered in this patient appears to be coincidental. Although recent ultrastructural study of a so-called reticulum cell sarcoma (microglioma) of brain has revealed similarities between the tumor cells of histiocytic medullary reticulosis and this glioma [17] the appearance of the brain tumor in this patient was, unequivocally that of an ependymoma.

Recognition of this disorder *intra vitam* represents a unique diagnostic experience for histiocytic medullary reticulosis [4 6 16]

Summary

A diagnosis of histiocytic medullary reticulosis was established intra vitam by bone marrow examination in 45-year-old white man with bone pain and anemia. Results of electron microscopy of the neoplastic histiocytes and ferrokinetic studies support the hypothesis that the anemia in patients with this disorder is due to the unavailability of iron stores resulting from the excessive erythrophagocytosis.

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Authors' address: Drs. Zbigniew A. Zawadzki, Carlos E. Peña and Edwin R. Fisher, Departments of Medicine and Pathology, Veterans Administration Hospital, University Drive C, *Pittsburgh, Pa. 15240* (USA)

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Institute of Pathology University of Kiel
(Director Prof. Dr K. Lennert)

A Case of Acute Leukemia with Pseudo-Pelger Cells Containing Auer Bodies

L. D. Leder

Cytological, cytochemical, and electron microscopic studies have shown that Auer bodies are pathological azurophilic granules [1 4 5 6] They do not occur in normal blood cells and hitherto have been described in acute and chronic myeloid leukemia, in myelo-monocytic leukemia, and in undifferentiated blasts of acute erythremic myelosis. They have never been found in cells of the lymphatic system. Therefore, Auer bodies prove a given hematological case to be neoplastic and of myeloid nature.

Pseudo-Pelger cells, on the other hand, are atypical granulocytes. They display rather dense and coarse chromatin and partial or complete lack of segmentation. In contrast to the true Pelger abnormality the pseudo-Pelger variety is not hereditary Pseudo-Pelger cells have been reported in various forms of myeloproliferative disorders [19 18 21 2, 3 20 8 9] Gunz and Burry [8] state that the presence of pseudo-Pelger cells is quite diagnostic for the myeloid variety of acute leukemia. Others, e.g Thiele [21] assume this to be merely an incidental finding

In the following report a case of acute leukemia is described. This case is well suited for a discussion of the diagnostic significance and the meaning of pseudo-Pelger cells in hematological disorders.

Case Report

Bone marrow and blood smears of 23 years old male suffering from acute leukemia were studied. The marrow was highly cellular There were predominantly small rounded blasts

with a narrow cytoplasmatic rim. With Pappenheim stain these blasts could hardly be differentiated from lymphatic cells (fig 1) Only few erythropoietic cells, megakaryocytes and granulocytic cells were present. The mature neutrophils partly displayed the pseudo-Pelger anomaly (fig. 2) Such cells together with blasts could also be observed in peripheral blood smears, but not during remission which excludes heredity

In addition to Pappenheim stain, the naphthol AS-D chloroacetate esterase method was performed [10] the smears were fixed for 30 min in formalin-methanol 1 9 Incubating medium 1 drop of 4% pararosanilin in 2 N HCl was mixed with 1 drop of 4% sodium nitrite in distilled water After 60 sec 30 ml of 0.1 M Michaelis buffer pH 7.62, were added. The pH was adjusted to 6.3 with 2 N HCL. 10 mg of naphthol AS-D chloroacetate, dissolved in 1 ml of N,N-dimethylformamide were added. The mixture was filtered. The smears were incubated for 30 min at room temperature, rinsed in tap water counterstained with hemalum and mounted in glycerin jelly Results Nuclei blue, positive elements bright red.

A small percentage of the lymphoid blasts exhibited a positive naphthol AS-D chloroacetate esterase reaction, showing various degrees of positivity This finding allowed an easy and definite identification of the condition as acute myeloid leukemia, because this esterase reaction is specific for the neutrophilic cell series and for tissue mast cells, the latter being of no significance in our case [7 10, 11 17].

In addition, some of the cells contained Auer bodies (fig. 3) According to Fazcron *et al.* [4] these bodies could be demonstrated quite distinctively by means of the naphthol AS-D chloroacetate esterase reaction. Some of the pseudo-Pelger cells also exhibited Auer rods (fig. 4)

Discussion

Our observation clearly shows that the NASDCl-esterase reaction can be used to great advantage for the classification of acute leukemia

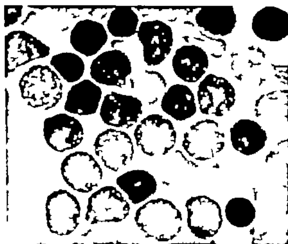


Fig 1 Bone marrow smear Predominantly small, lymphocyte-like cells. Naphthol AS-D chloroacetate esterase reaction (1 400)



Fig. 2. Pseudo-Pelger cells. Blood and bone marrow. Naphthol AS-D chloroacetate esterase reaction (×1,400)

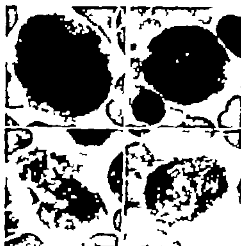


Fig. 3. Immature cells with Auer bodies. Naphthol AS-D chloroacetate esterase reaction (×1,400)

[11, 12, 14, 15, 16]. Thus, in the present case any doubt about the myeloid character of the lymphocyte like blasts could easily be eliminated by the demonstration of various grades of this esterase activity in some of these cells. The presence of Auer bodies proved this conclusion to be correct.

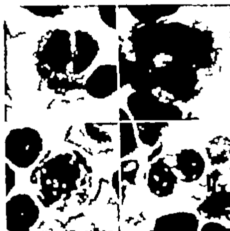


Fig. 4. Pseudo-Pelger cells containing Auer bodies. Naphthol AS-D chloroacetate esterase reaction (× 1400)

Furthermore, the existence of Auer rods in the pseudo-Pelger cells furnishes some information about the meaning of this abnormality in leukemias. The Auer bodies prove the pseudo-Pelger abnormality to be closely related to the leukemic process. Indeed, they represent pelgeroid paraleukocytes in the sense of ROHR [19]. In our case both paramyeloblasts and paraleukocytes were found. This observation supports ROHR's view that in leukemia differentiation as well as dedifferentiation takes place in the proliferating cell lines. This implicates that a given cell line (e.g. promyelocytes and myelocytes) may be affected by the leukemic process at various stages of maturation. The neoplastic condition obviously is not limited to – or beginning at – the stage of the myeloblast, as is believed by some authors. In consequence, atypical blasts and paraleukocytes are both derived from the same, originally normal cells. In many cases dedifferentiation with increasing anaplasia parallels progression of the disease. Thus, as we know, end stages frequently reveal almost pure populations of anaplastic blasts. In consequence, the appearance of blasts is a feature of the end stage, rather than of the initial stage of the leukemic process [19]. Detailed studies of myelo-monocytic leukemias have led us to the very same conclusion [13].

Finally, the occurrence of Auer bodies in pseudo-Pelger cells supports the view of GUNZ and BURRY [8], who claim that the demon-

stration of pseudo-Pelger cells in acute leukemia is quite suggestive of the myeloid nature of a given case. The report of Hayhoe *et al.* [9] is in good accordance with this interpretation. The latter authors described pseudo-Pelger cells in acute erythremic myelosis, acute granulocytic leukemia, and in acute myelo-monocytic leukemia. They never saw pseudo-Pelger cells in acute lymphatic leukemia. Due to his own observations this author also believes that the demonstration of pseudo-Pelger cells in hematological disorders, especially in unclassified acute leukemias, strongly supports the diagnosis of acute myeloid *leukemia and almost with certainty excludes acute lymphatic leukemia.*

Summary

A case of acute leukemia with pseudo-Pelger cells containing Auer bodies is reported. The significance of this observation and the diagnostic importance of pseudo-Pelger cells in hematological disorders are discussed. Some comments are given on the advantages of the naphthol-AS-D chloroacetate esterase reaction in relation to the classification of leukemias. *The appearance of pseudo-Pelger cells in the blood of patients with unclassified hematological disorders may be* first symptom of myeloid leukemia. I given case of acute leukemia the occurrence of pseudo-Pelger cells strongly supports the myeloid nature of the disease and almost definitely excludes lymphatic leukemia.

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Author address: Priv Doz. Dr L.-D. Leder, Pathologisches Institut der Universität, Hospitalstrasse 42, D-23 Kiel (Germany).

3rd International Transplantation Congress

The 3rd International Congress of the Transplantation Society will take place in the Netherlands Congress Centre, The Hague, from 7–11 September 1970. It will deal with the study of the different aspects of organ transplantation.

Organizing Committee: Prof. D. W. VAN BEKKUM, State University of Leiden; Prof. J. J. VAN LOGHEM, University of Amsterdam; Prof. J. J. VAN ROOD, State University of Leiden and Dr. H. BALNER, Radiobiological Institute of the Netherlands Organization for Applied Scientific Research (TNO).

Further information will be given by the secretariat, c/o Holland Organizing Centre, 16, Lange Voorhout, *The Hague* (The Netherlands).

XIII. Internationaler Kongress für Hämatologie, München 1970

Aus technischen Gründen musste der Termin für den XIII. Internationalen Hämatologen-Kongress geändert werden. Der Kongress wird vom *2.–8. August 1970* stattfinden. Alle jene, die noch keine vorläufige Anmeldung übersandt haben, werden gebeten, ihre Adresse dem Kongressbüro XIII. Internationaler Hämatologen-Kongress, Postfach 200, *D–8 München 12* mitzuteilen, damit Ihnen die Unterlagen zugesandt werden können.

XIIIth International Congress of Hematology, Munich 1970

For technical reasons, the date of the XIIIth International Congress of Hematology had to be changed. The Congress will take place from *August 2–8, 1970*. All those who have not yet registered provisionally are invited to inform the Congress Bureau, XIIIth International Congress of Hematology, P.O. Box 200, *D–8 Munich 12* (Germany), of their address so that details can be sent to them.

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German Academy of Sciences, Institute for Cancer Research, Experimental Section, Berlin-Buch

Studies on the Pathogenesis and Mechanism of Hematologic Diversification by Re-Isolation of the Myeloid Leukemia Virus (Graffi)

F. Fey

Preliminary studies have shown that the diversification of Graffi virus-induced leukemias depends essentially upon the type of starting leukemia used as virus source [8, 12, 13, 15]. This fact tends to a correlation between the marking of the various leukemia types and the cell form the virus used for infection has been obtained from them.

Organ filtrate studies using virus obtained from myeloid leukemias for infecting newborn mice have given first evidence about the pathogenesis of this diversification [6, 9, 14]. We started extensive experiments to determine the site of primary virus attack being important for the mechanism of diversification. Then the virus has been isolated from the place of formation at different intervals of time from the varying types of leukemia and was investigated for leukemogenic activity by bioassay.

Materials and Methods

Newborn random-bred mice of the inbred strain XVII Bln. were injected subcutaneously with the virus in form of cell-free filtrates from mature-cellular myeloid, paramyeloblastic, lymphatic or reticular leukemia. Five, 10 and 20 days after inoculation spleen, lymph nodes, bone marrow, thymus, liver and brain were removed from the treated young mice, cell-free preparation was made, and the filtrate diluted 1:100 in relation to fresh weight of the organ was re-administered to newborn XVII-mice. The treated mice remained with their mothers until puberty. Then they were separated according to sex. Control for the presence of leukemias followed at intervals. When leukemia occurred the mice were killed. The leukemia types were classified according to their blood picture and organ sections and, in some doubtful cases, according to criteria due to cytochemical reactions [10].

Results

Table I referring to myeloid starting leukemia shows the strong leukemogenic action of filtrates (except brain) prepared on the 5th day following infection. Strongest activity has been observed with spleen and liver filtrates having shortest latent periods, too. Organ filtrates *(except brain) prepared 10 days after infection show already maximum* leukemogenic action, the majority being myeloid leukemias. There is moderate diversification only the lymph node filtrate shows stronger diversification. The proportion of myeloid and reticular-mixed leukemias is high with 5 day filtrates and then continuously decreases with strongly leukemogenic organ filtrates of spleen and liver obtained 10 or 20 days following infection. Chloroleukemias increase in the same relation. In preparations made 20 days following infection, application of spleen filtrate induces 97 % myeloid leukemias, among them 77% chloroleukemias, whereas lymph node filtrates caused only 50% myeloid leukemias, among them 35 % chloroleukemias. Thus spleen filtrates from myeloid leukemias induce the strongest degree of identical reproduction.

Table II referring to a paramyeloblastic leukemia serving as virus source shows that only spleen filtrates have a distinct leukemogenic action at a relatively long latent period. Maximum leukemogenic activity is reached only by filtrates prepared 20 days after infection. Diversification is considerable almost all types of leukemias were induced. Myeloid and reticular mixed leukemias become strikingly rare. Spleen, bone marrow liver and lymph node filtrates cause predominantly myeloid leukemias. Thymus filtrates, however induce mainly lymphatic and reticular leukemias. The identical reproduction of the paramyeloblastic leukemia is as low as 10 %.

Table III presents the results of experiments with a lymphatic starting leukemia. In filtrates isolated 5 days after virus infection, a distinctly leukemogenic action of the thymus preparation becomes apparent which is expressed by a short latent period. In the same experiments a weaker activity is exhibited by spleen and bone marrow filtrates. Maximum inducing activity is caused by most of the organ filtrates produced 10 days after virus infection. This concerns predominantly thymus, spleen and lymph node filtrates. The diversification is strong so that almost all leukemia types were induced. Thymus and lymph node filtrates induce lymphatic and mixed forms by 50

Table I. Leukemogenic effect of cell-free organ filtrate prepared from mice infected with virus of myeloid leukemia origin

Organ filtrates prepared from		Number of mice	Number of leuk-emia	Percent	Latency days	Percent distribution of leukemia types										
						ma. my leuk.	im. my leuk.	chlo. leuk.	paramy bla. leuk.	my ret. leuk.	ret. leuk.	ret. ly leuk.	ly leuk.	ery bla. leuk.	ery my leuk.	not det.
Spleen		37	31	84	186	10	16	42		23	3	–	–	3	3	
	b	20	20	100	158	10	15	53	–	15	–	–	5	–	–	
		110	90	82	131	9	11	77		1		–	1	–	–	1
Lymph nodes		48	26	54	252	4	8	42		12	4	16	8	3	3	–
	b	32	30	93	158		17	57	7	7	3	3	–	3	3	
		71	60	84	130	7	10	35	10	21	3	8	2	–	–	4
Bone marrow		12	5	42	212	(20)	(40)		–	(20)	–	(20)	–		–	–
	b	11	10	91	172	30	10	40	–	20	–	–			–	–
		70	51	73	136	12	20	41	2	10	2	–	–	–	8	5
Thymus		18	9	50	224	(11)	(33)	(33)	(11)	(11)	–		–	–	–	
	b	6	6	(100)	139			(33)	–	(17)	(17)	(33)	–	–	–	
		26	25	96	139		20	44	–	16	–	12	–	–		8
Liver		31	19	61	185	21	21	16		21	16	–	–	–	–	5
	b	13	13	100	143	13	31	23	–	15	8	3	–	–	–	–
	c	79	58	73	137	5	29	31	–	7	3	9	–	2	3	9
Brain		46	9	20	228	(44)	–	(11)	–	(22)	–	(11)	(11)	–	–	
	b	7	3	(43)	174		–	(33)	–	–	–	–	–		(67)	–
		72	13	18	165	23	8	23		38	–	8	–	–		–

ma. my. leuk. mature myeloid leukemia, *im. my. leuk.* immature myeloid leukemia, *chlo. leuk.* chloroleukemia,
pamy. bla. leuk. paramyeloblastic leukemia, *my. ret. leuk.* mixed types of myeloid a. reticular leukemia,
ret. leuk. reticular leukemia (all leukemias originated in the RHS); *ret. ly. leuk.* mixed types of reticular and lymphatic leukemia,
ly. leuk. lymphatic leukemia, *ery. bla. leuk.* erythroblastic leukemia, *ery.-my. leuk.* mixed types of erythroblastic and myeloid leukemia.
a) Filtrates prepared 5 days, b) 10 days, and c) 20 days after virus infection.

Table II Leukemogenic effect of cell-free organ filtrates prepared from mice infected with virus of paramyeloblastic leukemia origin

Organ filtrates prepared from		Number of mice	Number of leukemia	Percent	Latency days	Percent distribution of leukemia types: ma. my leuk.	lm. my leuk.	chlo. leuk.	pamy bla. leuk.	my ret. leuk.	ret. leuk.	ret. ly leuk.	ly leuk.	ery bla. leuk.	ery my leuk.	not det.
Spleen		34	6	18	245	(33)	(33)	(17)	–	(17)	–	–	–	–	–	–
	b	29	21	72	169	–	10	48	14	10	–	10	4	–	4	–
	c	18	16	100	115	19	13	30	13	–	6	–	6	–	13	–
Lymphnodes		37	–	–	–		–	–	–		–	–	–	–	–	–
	b	33	20	61	172		5	70	5	5	5	–	10	–	–	–
	c	30	30	100	153	3	10	40	10	7	3	7	10	7	3	–
Bone marrow		28	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	b	23	16	70	146	6	–	50	6	6	–	6	20	–	6	–
	c	27	22	82	168	4	14	45	4	–	–	–	4	14	14	–
Thymus		39	1	3	186		–	–	–	–	–	(100)	–	–	–	–
	b	28	24	86	129		4	25	8	4	17	4	29	8	–	–
	c	32	30	94	127	–	10	17	7	3	17	3	20	10	10	3
Liver	a	32	1	3	318		(100)	–	–	–	–	–	–	–	–	–
	b	33	23	66	194	4	4	63	–	4	9	4	9	–	–	–
		24	21	88	170	–	19	42	5	5	9	5	5	5	5	–
Brain		27	–			–	–	–	–	–	–	–	–	–	–	–
	b	32	9	27	183	–	(11)	(78)	–	–	(11)	–	–	–	–	–
	c	25	10	40	140	–	–	60	–	20	10	–	10	–	–	–

) Filtrates prepared 5 days, b) 10 days, and) 20 days after virus infection.

Table III Leukemogenic effect of cell-free organ filtrates prepared from mice infected with virus of lymphatic leukemia origin

Organ filtrates prepared from		Number of mice	Number of leuk. mice	Percent	Latency days	Percent distribution of leukemia types: ma. my leuk.	lm. my leuk.	chlo. leuk.	pamy bla. leuk.	my ret. leuk.	ret. leuk.	ret. ly leuk.	ly leuk.	ery bla. leuk.	ery my leuk.	not det.
Spleen		29	4	14	201			(75)		–		–	(25)	–	–	–
	b	33	29	88	137		17	45		7	10	3	15	3		–
	c	33	29	88	120		7	48	–	15	3	7	10	3	3	3
Lymphnodes		39	2	5	200								(100)	–	–	–
	b	34	30	88	149		7	37		17	3	10	20	–	–	6
		31	28	90	111		7	11	4	7	11	18	36	–	3	3
Bone marrow		33	3	9	203			(33)	–		–	(33)	(33)	–	–	–
	b	32	25	78	150		24	36		4	4	12	20		–	
	c	24	21	88	118		24	19		24	5	9	9	9		–
Thymus		48	16	33	164		–	19		13	6	13	43	–	6	–
	b	41	36	88	139		11	28		3	8	17	22	3	3	3
		31	26	84	116		19	19	–	12	4	19	27	–		
Liver		17					–		–			–	–	–		–
	b	34	25	74	132		20	36		–	8	4	28	–	4	–
		33	28	85	144		11	32	4	11	4	17	14	–	7	–
Brain		31	1	3	240	–							(100)		–	–
	b	27	15	55	148	7	13	40	–	7	–	13	13	–	–	7
	c	26	8	31	170		–	(13)		(25)		(37)	(13)	(13)	–	

a) Filtrates prepared 5 days, b) 10 days, and) 20 days after virus infection.

Table 17 Leukemogenic effect of cell-free organ filtrates prepared from mice infected with virus of reticular leukemia origin

Organ filtrates prepared from		Number of mice	Number of leuk emis	Percent	Latency days	Percent distribution of leukemia types: my. my leuk.	im. my leuk.	chlo. leuk.	panmy bla. leuk.	my ret. leuk.	ret. leuk.	ret. ly leuk.	ly leuk.	ery bla. leuk.	ery my leuk.	not det.
Spleen	a	28	1	4	194	–		–	–	(100)	–	–	–	–	–	–
	b	33	7	21	191	–	–	(28)	–	(43)	(28)	–	–	–	–	–
	c	32	14	44	159	–	–	57		21	21	–	–	–	–	–
Lymph nodes		31			–	–	–	–	–	–	–	–	–	–	–	–
	b	26	9	35	187		–	(56)	–	(22)	(11)	(11)	–	–	–	–
	c	24	11	46	140		–	55	–	18	9	9	–	–	–	9
Bone marrow	a	36	3	8	186	–	–	(33)	–	(33)	(33)	–	–	–	–	–
	b	37	13	35	182		8	38	–	8	38	8	–	–	–	–
		27	9	33	157		–	(33)	–	(56)	(11)	–	–	–	–	–
Thymus	a	27	–	–	–			–	–	–	–	–	–	–	–	–
	b	40	28	70	142	–	–	43	–	7	28	–	–	4	4	14
		46	38	83	130	–	–	34	–	11	45	5	–	–	–	5
Liver	a	46		–		–	–	–	–	–	–	–	–	–	–	
	b	32	15	47	187	–	–	60	–	7	33	–	–	–	–	–
	c	25	12	48	148	–	–	33	–	–	50	17	–	–	–	–
Brain		32	2	6	215		–	(50)	–	(50)	–	–	–	–	–	–
	b	29		–	–	–	–	–	–	–	–	–	–	–	–	–
	c	27	3	11	142	–	–	(100)	–	–	–	–	–	–	–	–

) Filtrates prepared 5 days, b) 10 days, and) 20 days after virus infection.

whereas only 20% of these types were produced by myeloid organs. The identical reproduction in these target organs is 30–40%.

Table IV shows the results of organ filtrate experiments with a reticular starting leukemia. The primary leukemogenic action is weak and does not become manifest with filtrates produced 5 days after infection. Only thymus filtrates produced 10 days after virus infection have a strong leukemogenic action. Diversification is strikingly low. Lymphatic leukemias did not occur. It is astonishing that chloroleukemias are induced almost exclusively in the myeloid series. Reproduction of reticular leukemias is up to 50% in liver and thymus.

Discussion

As distinctly shown by the experiments, organ filtrates of animals inoculated with virus of a myeloid leukemia show maximum leukemogenic activity. Those animals treated with virus from a lymphatic leukemia follow in the second place. The lowest effect is obtained with organ filtrates of mice infected by virus of paramyeloblastic and reticular leukemias. The primary site of virus attack can be determined by the parameters of leukemia induction quota and latent period in series of experiments using virus re-isolated shortly after infection. The strongest primary action with virus prepared 5 days after infection is shown by filtrates of mice treated with virus of myeloid and paramyeloblastic leukemias and thymus filtrates of mice treated with virus of lymphatic leukemias. The primary effect of the virus in reticular leukemia in the bioassay becomes manifest only in thymus filtrates produced 10 days after infection. If the leukemogenic action is in direct proportion to the amount of active virus those organs showing the strongest primary leukemogenic action in the filtrate experiment should also contain the greatest amount of virus. Re-isolation of the virus shortly after infection exclusively comprises the newly formed virus at the primary sites of its formation. Target cells being preferentially infected and having first replication are different for viruses isolated from the hematologically different leukemia forms. Our studies have shown that target cells for the virus from myeloid and paramyeloblastic leukemias are localized preferentially in spleen, for the viruses from lymphatic leukemias in thymus, and for the virus from reticular leukemias predominantly in thymus and liver. Whereas the

primary replication proceeds in the specific target cells subsequent multiplication of virus may take place in a number of other cells of the organism. Megakaryocytes are of special importance in this regard. They are considered as essential sites of multiplication of murine leukemia viruses [1 4 5 17] A fundamental difference exists between specific target cells and all other cells in which a multiplication of viruses takes place. Only the target cells can be induced by the leukemogenic virus to undergo malignant transformation. Thus the viral leukemogenesis requires for its manifestation the active leukemogenic virus on the one hand and the specific sensitive target cell, on the other

In connection with splenectomy experiments [6] we referred to the role of the spleen in the pathogenesis of virus-induced myeloid leukemias. An extirpation of the spleen up to 5 days following virus application from mature cell myeloid leukemia reduces the leukemogenic incidence to about 20% as compared with shamoperated control mice. It is probable that splenectomy removes target cells in which primary replication of the virus occurs within the first 5 days and which, by the action of the virus, undergo transformation into malignant leukemia cells. Splenectomy after the 5th day following infection and virus administration from immature-cell myeloid leukemia induces a weaker effect and remains without any effect when using virus from reticular and lymphatic leukemias [6] These aspects have not been duely considered in splenectomy experiments made by other authors [11] so that their results cannot be compared with ours. As to irradiation induced myeloid leukemias of RF mice significant changes of the leukemia incidence after splenectomy have been also observed by UPTON *et al.* [18]

According to our findings our virus strain is inclined basically to induction of myeloid leukemias. This is manifested not only by the highest rate of identical reproduction, but is distinctly shown by the high proportion of myeloid leukemias induced by virus from hematologically different types. The identical reproduction of a given type of leukemia is induced, to a great extent, by the virus isolated from organs containing target cells sensitive to that virus. This conclusion is reasonably drawn by our findings using virus preparations from mature-cell myeloid leukemias. The fact that the virus re isolated from target organs induced only a low hematological diversification in bioassays in contrast to the virus formed outside of the target cell is an

essential finding in elucidating the mechanism of diversification. A further factor for the increased diversification and the correspondingly less identical reproduction is the use of virus from immature-cell leukemia, in our case paramyeloblastic leukemia. On the basis of our tentative hypothesis [8, 14] we assume that the virus produced in immature leukemia cells has less receptors from the mother cell due to the weaker specific character of the cell. This might be the reason of the reduced histotropy of the virus and the intense diversification of the leukemia types induced.

PASTERNAK and PASTERNAK [16] using MAP test were able to demonstrate Graffi virus indirectly 1 day following infection in spleen, liver and brain and 3 days after infection in thymus and lymph nodes, too. Spleen and liver homogenates were most active in producing antibodies. Least activity was found in thymus and brain. This suggests that the induction of antibody formation by the virus and the leukemogenic action of the virus are closely correlated. A marked leukemogenic activity of brain filtrates has been observed in our experiments only after infection with virus from lymphatic or paramyeloblastic leukemias. In experiments of BUFFETT *et al.* [2] brain filtrates prepared 1–2 weeks after administration of Ha/JCR virus had a leukemogenic action by 1 log lower as compared with filtrates from spleen, liver and kidney.

A part of leukemias occurring in our investigations represented mixed forms. The presence of a mixed form can be diagnosed easily by the different mode of infiltration of the leukemia component. CHIECO-BIANCHI [3] who administered Graffi virus to C57 Bl mice described the simultaneous occurrence of myeloid and lymphatic leukemias in an individual mouse. We could not confirm this observation, although we used much a greater experimental material.

Acknowledgement. We are indebted to Miss BRIGITTE ZELLER, Mrs. DAGMAR RICHTER, and Mrs. CHRISTINE GUTEMANN for their technical assistance.

Summary

Virus from hematologically different forms of GRAFFI virus-induced leukemia was re-isolated from the sites of formation at different intervals after application to newborn mice. By bioassay it was investigated for its leukemogenic activity. Organ filtrates of mice inoculated with virus from myeloid leukemias show highest leukemogenic activity followed by virus from lymphatic leukemia. Most intense primary action was found in spleen filtrates

of mice treated with virus from myeloid and paramyeloblastic leukemias as well as thymus filtrates from mice treated with virus from lymphatic and reticular leukemias. An identical reproduction of given leukemia type is preferentially induced by virus isolated from organs containing target cells specific for the virus. The particular role of the spleen in myeloid leukemogenesis is discussed.

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Author's address: Dr. habil. F. Fey, DAdW Institute of Cancer Research, Experimental Section, Lindenberger Weg 70, *1115 Berlin-Buch* (Germany)

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Department of Internal Medicine, University of Pavia, Pavia

Chronology of the Mitotic Cycle of Acute Leukemia Cells

E. G. Rondanelli, E. Magliulo, G. Pilla, F. Falchi and
G. M. Barigazzi

Since the demonstration by the stathmokinetic test of Astaldi and Mauri [1] that acute leukemia cells in the overall proliferate at a lower rate than normal granuloblasts, many other reports, based on radioisotopic techniques [2, 3, 4, 5, 6, 7, 8, 9, 10] have shown that in the majority of cases the production of acute leukemia cells may be inferior to that of normal granulocytopoiesis.

The purpose of this paper is to present some chronological values obtained by phase-contrast cine micrographic studies on proliferating acute leukemia cells.

Materials and Methods

Acute leukemia cells were obtained from bone marrow and peripheral blood of 10 acute (parahemocytoblastic) leukemia patients. Only those individuals were chosen in which the illness was diagnosed for the first instance. No previous specific treatment had been given. Only results obtained in those patients in which the leukemic process approximated a steady condition were employed. This was stated by the number of blasts circulating in peripheral blood remaining constant for at least one week after the study.

A part of bone marrow and peripheral blood samples was smeared and stained with May-Grünwald-Giemsa. The remaining particles of bone-marrow tissue were cultivated in vitro for 24 h and studied by phasecontrast cine-micrography according to a technique already described [11].

Mitotic indices (mitoses/1000 cells) were determined on bone marrow and peripheral blood smears by counting 10,000 cells for patient. Each 1000 bone marrow acute leukemia cells were further divided in two classes according to size: 'large' cells with a diameter > 13 µm, and 'small' cells with a diameter < 13 µm.

Results

The results of studies on panoptically stained smears of acute leukemia (AL) bone marrow and peripheral blood are reported in

Table I. Cytological studies on smears of bone marrow and peripheral blood from AL patients

Patients	1	2	3	4	5	6	7	8	9	10	Average ± S.D.
Mitotic indices (mitoses/1,000 cells) in bone marrow AL cells	1.0	6.5	1.3	4.6	2.4	0.5	2.8	3.9	3.5	5.2	3.17 ± 1.94
Mitotic indices (mitoses/1,000 cells) in periph. blood AL cells	0.0	0.1	0.0	0.2	0.1	0.0	0.0	0.1	0.0	0.3	0.07 ± 0.11
'Large' cells/ *1,000 AL cells* in bone marrow	53	301	77	295	164	259	433	165	138	320	220.5 ± *120.5*
'Small' cells/ 1,000 AL cells in bone marrow	947	699	923	705	836	741	567	835	862	680	779 ± 120.5
Mitotic indices of AL cells corrected for the presence of 'small' cells	18.8	21.5	16.8	15.5	14.6	1.9	6.4	23.6	25.3	16.2	16.06 ± 7.27

Table II. Distribution according to mitotic phase of 317 karyokineses counted in acute leukemia bone marrow smears

Mitotic phase	Prophase	Metaphase	Anaphase	Telophase
Absolute values	34	190	29	64
Percent values	10.72	59.93	9.14	20.18

tables I and II. The morphological development of AL cell mitosis is illustrated in figures 1 and 2.

The chronological values obtained by the phase-contrast cinemicrographic study of 20 AL cell divisions in the living state are reported in table III. As shown in table I, AL cells display a very low mitotic index in bone marrow. Furthermore, values very near to 0 are approached in peripheral blood. No mitoses were seen in 'small

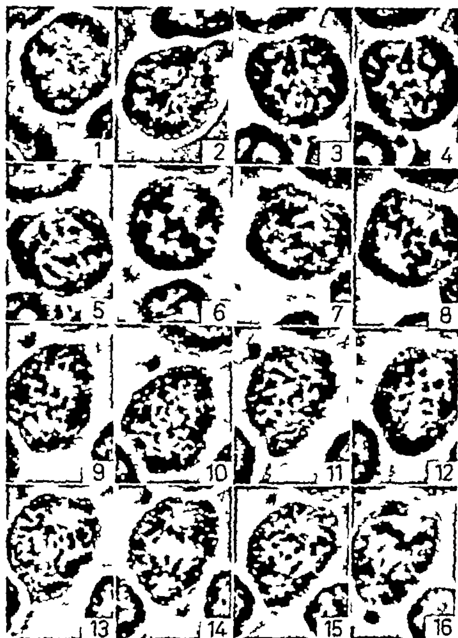


Fig. 1. Morphological aspects of mitosis in an acute leukemia paraheemocytoblast. Frames from phase-contrast time-record on living cells in vitro. 1 and 2 prophase; 3–5 metaphase; 6 and 7 *profile view of the equatorial plate; 8–16 anaphasic migration of daughter chromosomes* toward the poles, with some cytoplasmic bubbling in 14–16.

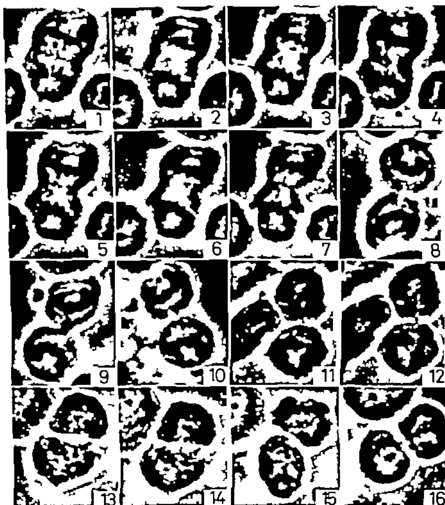


Fig. 2. Same cell as seen in figure 1 in the course of telophase. The photos show the development and deepening of cytoplasmic furrow until cleavage of the cell in two separated daughter units. The cell exhibits emissions of cytoplasmic bubbles along its contour. Note the bilobed aspect of the nucleus in one daughter cell.

blasts so that a correction was afforded to mitotic indices, due to the presence of this non proliferating or at least very slowly proliferating fraction of cell population. Consequently a marked increase in mitotic indices resulted in the majority of cases, with an average

Table III. Duration of mitosis and its phases (min/sec) in AL cells studied *in vitro* by phase contrast cinemicrography

Mitosis No.	Prophase	Metaphase	Anaphase	Telophase	Total mitotic time
1	9'52	38'40	5/19	13'42	67/33
2	9'36	30'06	11/08	22/14	73'04
3	4'28	53/10	5/03	16'78	81/11
4	9'19	37'01	5/22	19/50	71/32
5	8/50	40'20	7/41	16/11	73'02
6	9,26	44/13	4/22	11/38	69'41
7	10/11	52'06	9/12	22/02	93/31
8	8'00	57/15	2/'0	12/39	80,14
9	9'56	43/58	8/37	20/40	85/11
10	10'31	49/19	10'20	13/40	83'30
11	5/38	48'36	7/09	20'02	81/25
12	7/22	40'49	5'56	21/34	75'41
13	9'02	54/16	6/16	12/03	81/37
14	8/33	38/54	8'59	10'34	67/20
15	11/50	42/39	10'50	20,45	86,04
16	7/45	33'08	4'41	22/39	68'33
17	7/34	48'00	7'06	17/78	80'58
18	4'20	35/31	6/48	19'54	67/33
19	12'27	47'00	5'24	21/43	86'34
20	12/46	36'33	8/46	16'00	74'05
Mean	8'53	43'46	7'07	17/38	77/25
SD	1/11	7/39	2/21	4'04	7/38

value being approximately fivefold the mean of mitotic indices of the whole leukemic cell population (proliferating + non proliferating cells) A large proportion (59.93) of mitotic figures was in meta phase (table II) A minor number (20.18%) were in telophase followed by prophase (10.72) and anaphase (9.14)

Mitosis in leukemia cells studied by phase-contrast cinemicrography had a mean duration of 77 min 25 sec ± 7 min 38 sec (table III) The major part of average karyokinetic time was spent in metaphase (about 56 of mitotic time) and at a lesser degree in telophase (2?) and then in prophase (11) and metaphase (9) For each mitotic phase the percent fraction of mitotic duration appeared well correlated with the relative incidence among mitotic figures in bone marrow smears.

Discussion

Evidence is being accumulated that acute leukemia cells are not provided of high proliferative potentials [1–10]. In such an instance, the comparison is made with normal granulocytopoietic cells, so that more extensive informations on time parameters of mitotic cycle of both normal and leukemic cells are urgently needed.

In a previous report [11] we made an attempt to characterize the chronology of normal granulocytopoietic proliferation. The time estimates were performed by combining informations derived from mitotic indices in bone marrow smears and mitotic durations directly determined on living cells.

A similar series of data on acute leukemia cells is presented in this paper basing on the assumption that possible influences due to the *in vitro* environment would exert their action to the same extent on both normal and leukemic cells. Furthermore the chronological data previously presented fit well with kinetic data obtained by radioisotopic techniques. Thus it is assumed that our chronological data were approaching the *in vivo* situation more closely than those obtained by other *in vitro* methods.

In the present report the low mitotic index of acute leukemia cells [1, 12, 13, 14, 15] is confirmed. Extremely rare mitoses were found in peripheral blood, so that, for sake of simplicity circulating AL cells were considered non proliferating elements and AL cells were assumed to be produced almost exclusively in bone marrow. The great majority of mitoses in bone marrow were confined to large blasts.

As the mitotic index is given by the ratio mitoses/cells capable of division mitotic indices were corrected for the presence, in the denominator, of the 'small' cells not capable of division. It is interesting enough that the corrected mitotic indices ranged from a minimum of 1.9‰ and a maximum of 25.3‰ with an average of 16.06 ± 7.27‰ thus approaching in the majority of cases that of normal myeloblasts (23.64 ‰) and being even higher than that of normal promyelocytes (13.28‰). If we could make a further correction of mitotic indices for the presence of non proliferating cells also in the 'large' blast population, probably values superposable to those of normal myeloblasts would be attained.

It is well known that the mitotic index is a measure not only of cell production, but also of relative mitotic duration [1]. The chrono-

logical studies performed in the living afforded values of AL cell mitotic time (77 min 25 sec) much higher than those previously obtained in normal granulocytopoietic cells (44 min 47 sec in myeloblasts, 53 min 59 sec in promyelocytes and 65 min 23 sec in myelocytes) thus confirming also the reports by BOLL *et al* [14 15]

As already mentioned [16, 17] mitosis in undifferentiated stem cells lasts longer than in differentiated parenchymal cells. This fact *shows that AL cells behave as an undifferentiated population also at* the level of mitotic duration. Furthermore the emission of cytoplasmic bubbles during AL cell cytokinesis (fig 2) constitutes an almost exclusive morphological characteristic of undifferentiated hemopoietic cells [17] A good correlation was found between the fraction of mitotic time attributable to each karyokinetic phase and the frequency (over 100 mitoses) of karyokinetic figures pertaining to the same phases in bone marrow smears, the latter frequency thus being a good marker of relative mitotic phase duration.

The finding of a mitotic time longer than normal may be discussed in terms of the overall proliferative potentials of AL cells. However as KILLMANN recently pointed out [9 10 18] a great many assumption must be made in order to compare normal and leukemic hemopoiesis on the basis of mitotic (or labeling) indices and durations of mitosis (or of other phases of mitotic cycle)

First of all AL cell proliferation is characterized neither by a steady-state condition nor presumably by an 'orderly timed progression The proliferative potentials are very variable in relation to different patients and to different phases of the illness in the same patient [19 20, 21] Cell death rates and the exact proportion of cells maturing without division, or of cells dividing in extramedullary sites, are unknown. Furthermore the nature (multiplicative or stem cell) of proliferating compartments has not yet been defined though GAVOSTO [19] recently suggested the existence of an unrecognized stem cell compartment feeding into the multiplicative blast line. It is not known whether apparently non dividing cells are able to resume proliferative activity under certain stimuli.

We may accept that in the patients studied by us a steady condition was approached at least for the relatively brief duration of the experiments, and that AL cell production was almost exclusively or prevalently confined to 'large blasts in bone marrow Small blasts could well constitute a non proliferating maturative-like subpool.

If 'large' blasts were the stem-cells of the system or at least cell entry from other unrecognized compartments is small AL cell proliferation could be approached to that of a proliferating pool initiated by a stem cell and terminated by a heteromorphogenetic division [18] i.e. the last division of 'large' blasts giving rise to two cells ('small' blasts) morphologically different from their mother-cell. This could be the case in figure 2 where one of daughter cells is provided with a bilobed nucleus.

This is a very hypothetical construction which obviously does not correspond exactly to the real conditions, but it is the only one which allows to make some computations on the bases of mitotic indices and mitotic times, even if the latter though rather physiological were obtained *in vitro*. For a full discussion of the limits to the application of such indices for estimating proliferative time parameters the paper of KILLMANN *et al.* may be consulted [18]. Suffice it to say that the computed weighted average generation time for the 'large' blast compartment should be 80 h. Such a generation time might correspond to transit time if AL 'large' blasts undergo only one division and phase transition ('large' becoming 'small') corresponds to mitosis. This does not seem to happen in real conditions [21]. Otherwise only a maximum transit time could be computed by $N/K_{out} = N/K_M$ where N = cell number in the population K_{out} cell exit rate and K_M = mitotic rate, the latter being computed in 12.44/1 000 'large' blasts. The maximum transit time should then correspond to 284 h, that is 11 days. In a previous report we presented some time estimates for normal granuloblasts [11]. The weighted average generation time for all granulocytic precursors capable of division was less than 90 h and probably very near to 60 h. The transit time of myeloblasts was 31 h with a total transit time of granulocytopoietic cells of 127–240 h. These values are rather shorter than those computed for AL cells.

We may not state that the structure of the proliferating pools of normal granuloblast and acute leukemia cells is comparable. However such data prompt us to consider that generation time and transit times of 'large' leukemia blasts are longer than those of normal granuloblasts considered in the whole. If the comparison was to be made between AL 'large' blasts and their nearest normal counterparts, the myeloblasts, or between the total blast population ('large' + 'small') and the total normal granulocytopoietic population an even lower than normal proliferative potential could be assessed for AL

cells. Our assumptions are in good agreement with the concepts recently expressed by KILLMANN [10] in a review on AL cell prolifera tion. The data of previous authors show that at least in the majority of adult cases the generation time of leukemic blasts is not only longer than that of normal myeloblasts but also than that of the whole normal granulocytopoietic precursors. This confirms what has been previous stated by early investigations by the stathmokinetic test [1]

Summary

Samples of bone marrow and peripheral blood from 10 patients with acute parahemocytoblastic leukemia were studied by determining mitotic indices, relative phase frequencies, in vivo duration of mitosis and its phases, and by computing the average generation time and transit time. The results are in accord with the thesis that the proliferative activity of acute leukemia cells is lower than that of normal granulocytopoetic cells.

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Authors' address: Prof. Dr. E. G. RONDANELLI, Prof. Dr. E. MAGLIULO, Prof. Dr. G. PILLA, Dr. F. FALCHI and Dr. G. M. BARBARANI, Department of Internal Medicine, University of Pavia, *Pavia* (Italy).

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Department of Clinical Pathology, Royal Victoria Hospital, Belfast

Distribution of Amino Acids in Urine, Plasma, Leucocytes and Erythrocytes of Leukaemic and Normal Subjects

MAUREEN B. LEE, C. DENISE BOLGER and J. M. BRIDGES

A variety of regimes, based on modification of the dietary amino acid content, has been advocated in the management of acute leukaemia. ALLAN *et al.* [1] gave supplements of phenylalanine and tyrosine to 5 children including one in whom it was the only therapy and in whom the total white and blast cell counts fell. HALIKOWSKI *et al.* [6, 7] and HILSON [8] gave diets with low purine and low animal protein content in addition to conventional therapy and they felt that these were associated with better responses. The relative value of these various regimes, as all the authors emphasise, must await further larger and more rigorously controlled clinical trial. Evaluation of this type of therapeutic approach would be helped if there was knowledge of how the leukaemic subjects differed from normal with regard to their amino acid metabolism.

In a previous study [11] we reported on the urinary amino acid pattern in leukaemic and normal subjects and in this report we extend these studies to an analysis of the amino acid composition of normal and leukaemic plasma, red and white cells.

Materials and Methods

Fifty healthy hospital staff served as controls and 36 patients with leukaemia (20 acute, 8 chronic lymphatic and 8 chronic granulocytic) were studied. In all patients the clinical, peripheral blood and bone marrow findings were typical of the respective types. Patients were at various stages of their illness and were receiving treatment with steroids and cytotoxic agents. Blood and urine samples were taken without reference to previous protein intake and at least 2 sets of specimens were obtained from each patient. Siliconised glassware was used throughout the preparation of blood specimens.

Blood samples (10 ml) were anticoagulated by the addition to diaminoethane-tetra-acetate (2.0 mg/ml EDTA) After centrifugation at 900 g for 10 min, 2 ml of plasma was removed and replaced by 2 ml Intradex (Glaxo Laboratories). This caused rapid sedimentation of the red cells and some 30 to 60 min later the supernatant plasma containing many white cells, but only a few red cells was removed. The cells were deposited by centrifugation at 100 g for 10 min and the supernatant discarded. By the addition of 5 ml of ice cold water remaining in contact with the cells for not more than 30 sec, the red cells were lysed. Then 5 ml of a 1 percent solution of EDTA in hypertonic saline buffered with glyoxaline (pH 7.0) was added [7]. The cells were resuspended and washed twice in isotonic buffered saline (1% EDTA, pH 7.0) and finally resuspended in 1 ml. The white cell count was carried out and the suspension was homogenised by sonication for 2 min.

Red cell haemolysates were prepared from red cells washed three times in normal saline and then made up to give a final PCV of 53 % with distilled water. Haemolysis was completed by 3 cycles of freezing and thawing. Plasma, the red cell and the white cell homogenates, were filtered to dryness in a Sartorious membrane filter over an ice bath and the free amino acids were collected in the filtrate. Urine and the filtrates were desalted by electrodialysis, using the Baird & Tatlock ion exchange apparatus [19–22]. 150 µl of plasma and red cell homogenates, volumes of white cell homogenate to correspond to an extract from 10,000 cells (60–150 µl) and a volume of urine corresponding to a total non-protein nitrogen of 250 mg (50–200 µl) were used for chromatography.

The chromatographic method was as previously described [11]; 8 mm chromatography paper No. 541 placed on racks [4] were developed in 25% phenol solution with ammonia added. The second solvent, run at right angles to the first, was saturated 2, 4–2, 5 lutidene (Yorkshire Tar Distillers). The amino acid spots were identified by a comparison with a pre-constructed map of known amino acids. Spot densities were scored from 1–10 by comparison with 5 µl of α-amino-octanoic acid (scored 5) which was run with each batch of papers. The score, assessed by the same person throughout the study, was presented as the average obtained by dividing the total value for each of the amino acids by the number of specimens in each group. The numbers thus obtained for each of the 16 amino acids in the leukaemic group and in the control group were compared.

Results

The results of the urine, plasma, white cell and red cell chromatograms from the control and leukaemic subjects are shown in tables I–IV.

In the urinary chromatograms the leukaemic groups show very similar patterns, apart from the low methyl histidine in chronic granulocytic leukaemia and the low serine in chronic lymphatic and chronic granulocytic leukaemia. Comparison of all leukaemic subjects with the controls showed that in the former glutamic acid and methionine were increased, whereas valine was decreased.

The amino acid levels in the plasma from the leukaemic patients were very similar, the only exception being the serine level which was lower in the chronic granulocytic and lymphatic groups. Compar

Table I Amino acid distribution in urine

	Arg Lys Orn	Prol	Meth Hist	Hist	Glut NH	Asp	Glut acid	Gly	Ser	Ala	Threo	Meth	V I	Leuc	Tau	Tyr	Try	Cyst
Control	0.3		0.52	1.6	2.4	2.0	0.9	3.1	2.2	2.3	0.1	0.1	0.8	0.9	2.4	0.7	–	–
Acute leukaemia	0.78	0.00	0.33	1.3	2.6	2.0	2.2	3.3	1.8	2.9	0.65	1.4	0.4	0.65	2.1	0.3	0.1	0.2
Chronic granulocytic leukaemia	0.65	0.01	0.03	1.2	2.9	1.9	2.0	2.9	1.1	2.4	0.3	1.4	0.3	0.8	1.5	0.3	–	0.02
Chronic lymphatic leukaemia	0.4	0.03	0.3	1.1	2.3	1.8	1.9	2.6	0.63	2.8	0.25	2.6	0.2	0.4	1.8	0.2	–	

Table II Amino acid distribution in plasma

	Arg Lys Orn	Prol	Meth Hist	Hist	Glut NH	Asp	Glut acid	Gly	Ser	Ala	Threo	Meth	V I	Leuc	Tau	Tyr	Try	Cyst
Control	1.18	2.9			3.8	1.1	2.1	3.1	1.8	3.5	2.83	1.2	4.3	3.8	1.1	0.54	–	
Acute leukaemia	1.3	1.7		0.12	4.8	1.6	1.7	2.2	1.4	4.0	0.75	1.1	3.7	3.7	0.25	0.74	–	0.41
Chronic granulocytic leukaemia	1.8	2.1		0.04	5.1	1.7	2.4	2.6	0.7	4.8	0.9	0.8	3.9	4.1	0.3	0.9	–	0.7
Chronic lymphatic leukaemia	1.59	1.5		0.04	3.8	1.4	2.1	2.5	0.83	4.1	1.0	0.9	3.5	3.2	0.2	0.7	–	–

Table III Amino acid distribution in white cells

	Arg Lys Orn	Prol	Meth Hist	Hist	Glut NH_2	Asp	Glut acid	Gly	Ser	Ala	Threo	Meth	Val	Leuc	Tau	Tyr	Try	Cyst
Control			–		2.0	2.2	2.1	1.7	1.4	2.3	0.1	0.15	1.2	1.5	6.0	–	–	–
Acute leukaemia	0.64	0.2		0.06	1.8	2.2	2.7	1.7	1.1	3.0	0.84	0.4	1.7	2.3	3.4	0.16	–	–
Chronic granulocytic leukaemia	0.91	0.28		0.41	1.46	1.7	1.5	2.4	2.2	3.5	1.2	0.23	2.3	3.5	2.2	1.0	–	
Chronic lymphatic leukaemia	1.5	0.08	–	0.21	0.91	1.3	2.3	2.2	1.8	2.9	0.68	0.33	1.4	2.3	2.4	0.20	–	0.12

Table IV Amino acid distribution in red cells

	Arg Lys Orn	Prol	Meth Hist	Hist	Glut NH_2	Asp	Glut acid	Gly	Ser	Ala	Threo	Meth	Val	Leuc	Tau	Tyr	Try	Cyst
Control	0.06	0.08	–		3.0	5.5	4.0	2.2	2.2	3.0	0.58	0.06	1.3	1.2	0.83	–	–	–
Acute leukaemia	0.35	0.1		0.2	2.2	3.1	2.5	1.8	1.0	2.1	1.5	–	0.8	0.95	0.3	–	–	–
Chronic granulocytic leukaemia	0.8			–	2.2	3.1	2.5	1.8	1.0	2.1	0.3	–	0.85	0.9	0.3		–	
Chronic lymphatic leukaemia	0.26				2.1	4.6	2.4	2.0	0.8	2.2	0.2	0.06	0.8	0.9	0.46		–	–

ed with the normal, the leukaemic plasma had less threonine and taurine and apart from traces of histidine not found in the controls, no significant increase in any amino acid was noted.

Analysis of the chromatograms prepared from the white cell homogenates showed that within the leukaemic groups there was a great deal of variation and no overall pattern could be deduced. All leukaemic groups showed an increased threonine and a decreased taurine whilst small amounts of arginine lysine, proline, histidine and tyrosine were present which were not seen in the controls. In the chronic lymphatic group glutamine and aspartic acid were low whilst in the chronic granulocytic group serine valine and leucine were increased relative both to the other types of leukaemia and the controls.

The amino acid patterns from red cells in the leukaemic groups were very similar except that in acute leukaemia the threonine level was elevated Comparison of the leukaemic with normal red cells showed increased arginine and lysine, whereas the glutamic acid serine and taurine values were decreased.

Discussion

The finding of high urinary methionine and decreased valine in leukaemic subjects is similar to that of a previous study [11] The reason for this is not clear but it does not follow from changes in the plasma, which in the leukaemic subjects showed that the methionine and valine levels were similar to the control values. In the material here studied the levels of plasma taurine and threonine were decreased, but the other amino acids were normal Kelly and Waisman [10] in a study of the plasma amino acids of leukaemic subjects also found threonine to be lower than normal In all types of leukaemia they found glutamic acid to be elevated as finding also reported by other workers [20 21] Rouser [16] found glutamine levels to be very low in the plasma of leukaemic patients.

The red cells of our leukaemic patients showed a decreased level of glutamic acid, serine and taurine. Rouser et al [18] found red cell glutamic acid to be low in all leukaemic patients and in some cases glutamine and aspartic acid were increased McMenamy et al [13] found the glutamic acid and serine content of red cells from leukaemic patients to be increased

As would be expected, the most striking changes in this study were found in the amino acid content of the white cells. We found the arginine lysine proline, histidine and threonine increased and the taurine level decreased in all leukaemic groups, whereas only with the chronic myeloid leukaemic was the aspartic acid content low decreased taurine was also found in the leukaemic plasma and red cells. NOUR ELDIN and WILKINSON [14] reported the taurine content of leukaemic cells to be lower than normal, whereas IYER [9] found this amino acid to be significantly elevated in leukaemic leucocytes.

In this type of study as with many involving leukaemic white cells, it is not possible to obtain satisfactory control material and thus one is comparing primitive leukaemic cells with normal mature cells. In our method we studied the extract from the same number of white cells in each subject, but for the leukaemic this probably represented a greater cellular volume, and the influence of this cannot be ascertained From our results, and those of other workers, no discernible patterns involving any particular amino acid emerges for any specific type of leukaemia. It is interesting to note that many of the changes were similar for the various types of leukaemia and this has also been noted by other workers. The interpretation of this type of material is hindered by the fact that the level of plasma amino acids may vary throughout the day [23] Changes in amino acid patterns may also depend on protein intake [5 12] and certain complications of the disease and therapies may of themselves, cause changes [3 15 17 24] Some of our patients were on steroid therapy were having blood transfusions or a variety of cytotoxic agents if material is limited to patients examined prior to therapy then the numbers available would be very low A further difficulty is in the method of separation of amino acids by chromatography and the recording of the results as minor variations in techniques may lead to substantial changes in the results obtained. It would seem that the limitations of this type of study are such that it is unlikely that our understanding of the nature of the leukaemic process will be enhanced by them.

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Summary

Amino acid distribution in urine, plasma, white cell homogenate and red cell haemolysate from 36 patients with various types of leukaemia was compared with 50 control subjects. The patterns within the leukaemic groups were rather similar and, as between control and leukaemic groups, there were only minor differences.

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Authors' address: Mrs. Maureen B. Lee, C. Dennis Bolmer and Dr. John M. Bridges, Department of Clinical Pathology, Royal Victoria Hospital, Belfast (Northern Ireland)

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Hematology Division, University of Nebraska Medical Center Omaha, NB

Suppression by RNA of Human Lymphocyte Transformation

P. G. Rigby and D. M. Johnson

RNA from yeast added to normal human lymphocyte cultures has been shown previously to inhibit cell proliferation of phytohemagglutinin (PHA) stimulated cells [1–3]. The mechanism by which this suppression takes place and the potential sources and kinds of RNA concerned have not been determined [1–4]. A considerable amount of literature is available regarding the nature and action of RNA, including the kinetic study of RNA metabolism in lymphocyte-type cells as well as other biological systems [5–11]. The purpose of this paper is to investigate further the amounts and types of RNA which will produce suppression of human lymphocyte transformation (HLT).

Materials and Methods

The method of lymphocyte culture is based on that reported by Bach and Hirschhorn [12] and later by Johnson *et al.* [1, 3] from this laboratory. Heparinized human blood was allowed to sediment at 37° C in screw cap glass tubes for 3 h. Supernatant plasma was removed and 1–2 million cells in plasma were pipetted into flint glass prescription bottles containing 3 ml of medium (MEM Spinner media, supplemented with 20% fetal calf serum, penicillin, streptomycin, and L-Glutamine). The basic suspension was varied by the addition of *phytohemagglutinin (PHA) 0.02 ml/ml culture medium (General Biochemicals, Lot No. 651 601).*

Yeast RNA at varying concentrations from 0.03 to 30.0 mg/culture (Mann Research Laboratories, Inc., Lot No. L–1941) was utilized. Polyuridylic acid (Mann Research Laboratory Inc. Lot. No. T 3135) was obtained and utilized in the tissue cultures as indicated.

The suppression of lymphocyte growth and transformation was observed by cell counts on the 1st, 4th, and 7th days, using Cetrimide (1:10 dilution in 1% acetic acid concentration, 5 mg/ml) to avoid the clumped lymphocytes seen with the PHA incubation [13]. Cell cultures were done in triplicate and counts in duplicate. Transformation was suppressed

Table I. HLT suppression with RNA titration and effect of relyophilization (all cultures have phytohemagglutinin)

Exp.	Test materials	Amount of RNA mg/culture	Cell No. Mean ± SE × 10⁶	p
A	Control	none	1.55 ± 0.12	
	Yeast RNA	30 0	0.11 ± 0.04	< 0.01
		0.3	0.70 ± 0.03	< 0.05
		0.03	1.28 ± 0.1	> 0.05
		0.003	2.13 ± 0.31	< 0.01 (increased)
B	Control	none	1.93 ± 0.47	
	Yeast RNA	3.0	0.06 ± 0.03	< 0.01
		0.9	0.49 ± 0.08	< 0.01
C	Control	none	1.89 ± 0.06	
	Yeast RNA	0.9	1.09 ± 0.10	< 0.01
		0.3	0.64 ± 0.05	< 0.01
D	Control	none	2.89 ± 0.29	
	Relyophilized yeast RNA	3.0	0.48 ± 0.09	< 0.01
	Yeast RNA	3.0	0.26 ± 0.02	< 0.01
E	Control	none	3.96 ± 0.29	
	Yeast RNA	3.0	2.50 ± 0.06	< 0.01
	Relyophilized yeast RNA	3.0	2.25 ± 0.18	< 0.01

when the counts of all cells in the culture on the 7th day were significantly reduced from control levels (table I) The morphological evaluations using Wright' and/or Acridine Orange staining were entirely consistent with the suppression of HLT noted on the basis of cell counts, showing consistently lower percentage of transformed cells with fewer counts as previously reported [1 3].

Results

(1) Suppressed HLT occurred with yeast RNA in amounts of 0.3 0 9 3 0 and 30 0 mg of RNA/culture as seen in table I

(2) Suppressed HLT occurred with yeast RNA after relyophilization (table I)

(3) Polyuridylic acid, simulating artificial messenger RNA, did not suppress HLT in PHA stimulated cultures in doses of 0.01 to 2.0 mg/culture. However a significant reduction in cell numbers was observed with polyuridylic acid alone (table II)

Table II. The effect of polyuridylic acid on HLT

Exp.	Test material	Amount of RNA mg/culture	Cell No. Mean ± SE $\times 10^6$	p
A	Polyuridylic acid (without PHA)	none	0.60 ± 0.01	
		1.33	0.41 ± 0.03	< 0.01
		0.5	0.55 ± 0.11	0.02
		0.05	0.52 ± 0.04	> 0.05
		0.005	0.75 ± 0.06	< 0.01 (increased)
B	Polyuridylic acid (with PHA)	none	2.18 ± 0.61	
		1.33	3.09 ± 0.21	> 0.05
		0.5	2.16 ± 0.16	> 0.05
		0.05	1.35 ± 0.16	> 0.05
		0.005	2.67 ± 0.59	> 0.05

(4) The finding of a significant increase in cell numbers when small doses of RNA or polyuridylic acid were utilized may indicate a possible additional stimulating effect.

Discussion

RNA from yeast, before and after relyophilization, has been shown to decrease the transformation of lymphocytes in tissue culture stimulated by PHA. The possibility that a contaminant is responsible for this effect is reduced by the observations that relyophilized RNA is effective and that increasing doses of RNA give increasing suppression. Polyuridylic acid, simulating an artificial messenger RNA, did not suppress PHA stimulated HLT though a reduction in cell count was observed without PHA. Further study may show whether the suppressive effect is encountered with other kinds or fractions of RNA.

An understanding of the specificity of PHA and its action of the human lymphocyte has not been fully elaborated at this time. The site of RNA action in these studies is unknown. RNA has been reported to be on the surface of human cells, as well as in the cytoplasm and nucleus [14–16]. RNA is known to be incorporated into living cells in a number of experimental systems [2, 17]. The effect of RNA on

lymphocytes when PHA is not added is not as dramatic, but may show some suppression [9]. In the present study polyuridylic acid showed some effect without PHA. The apparent stimulation of HLT by small amounts of yeast RNA (with PHA) or poly U (without PHA) requires further study for satisfactory explanation.

The relationship between suppressed HLT by RNA and altered lymphocyte function can be correlated with *in vivo* transfer experiments using RNA in animals [18, 19]. RNA extracted from Ehrlich ascites tumor given at a dose of 4 mg/day for 8 days, as well as that extracted from white Swiss mouse liver (same dosage) have been shown to delay skin allograft rejection in mice (as well as yeast RNA) [18, 19]. The allografts were from C57/Black mice given to white Swiss mice; the normal rejection time was 9.5 days and the delays were to 12.5 and 11.4 days ($p = < 0.01$) respectively [18, 19].

The findings of suppressed human lymphocyte transformation *in vitro* and delayed skin allograft rejection *in vivo* support the hypothesis that biological interference with immunologic function is possible with RNA [20].

Summary

Human lymphocyte transformation (HLT) with PHA was suppressed by the addition of RNA in tissue culture. Polyuridylic acid, simulating artificial RNA, did not suppress HLT in similar dosages in PHA stimulated cultures. The relationship between suppressed HLT by RNA and altered lymphocyte function has been correlated with *in vivo* transfer experiments using RNA in animals.

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Authors address Prof Dr P G. Rigby and Dr D. M. Johnson, University of Nebraska Medical Center Omaha, NB 68105 (USA)

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Institute of Medical Pathology (Director Prof. A. Beretta Anguissola) University of Turin, Turin

The Starch Gel Quantitative Electrophoresis of Haemoglobin as Screening in the Thalassaemia Syndromes

G Ricco E. Gallo U Mazza, G Bianco and V Prato

The diagnosis of Cooley's anaemia does not present any difficulties, as the illness develops with clear clinical symptoms and typical haematological and biochemical characteristics. On the contrary in some thalassaemia trait carriers the diagnosis cannot be easy i.e when striking haematological and clinical features are absent. In these cases several tests, which are often linked to subjective interpretation are required. The authors who have dealt with the problem of the screening of thalassaemia agree in recognizing that none of these tests by itself enables one to detect unmistakably all the carriers [2 3 5 9] A simple method has recently been perfected which allows one to dose accurately all the haemoglobin fractions obtained by starch gel electrophoresis [6] By this method the value of each haemoglobin fraction in a sufficiently large number of thalassaemia heterozygous patients has been determined these values were compared with those drawn from a large number of clinically and haematologically normal subjects and with those of subjects suffering from haematological disorders of different kinds, in order to establish whether the quantitation of the haemoglobin fractions obtained by means of starch gel electrophoresis can profitably be used, even alone in the screening of thalassaemia.

Materials and Methods

The investigation was carried out on the following subjects (1) 200 healthy and haematologically normal subjects of ages varying between 15 and 75 years (2) 195 heterozygous

carriers of β- and $\beta\delta$-thalassaemia with microcytic anaemia and characteristic somatic stigmata. These patients of ages varying between 7 and 68 years came from the Po delta, from Southern Italy and the Italian islands only two of them originated from Piedmont; (3) 25 genetically certain carriers of thalassaemia trait there did not present thalassaemic somatic stigmata, but thalassaemia was present in their families and was transmitted by them to their children. Their mean haematological data were as follows Hb 13.9% (±0.83) RBC 4.70 (±0.26) × 10^6/mm³ MCH 29.62 pg (±2.15) PCV 42.48% (±1.57) MCV 91.32 μm³ (±1.35) There was no increase of bilirubin and red blood cell osmotic fragility was in most cases in the normal range (4) 4 subjects suffering from sickle-cell thalassaemia; (5) 67 subjects suffering from anaemias of different types.

Haemoglobin, erythrocyte count and hematocrit were determined in all the subjects from these values mean corpuscolar haemoglobin concentration and mean corpuscolar volume were calculated. In the thalassaemic patients and in their relatives red blood cell osmotic fragility in hypotonic solutions was determined by means of the method of Prato et al. [4] which enables one to determine the quantitative fragility curve. In the cases where the diagnosis was doubtfull reticulocyte count and sideraemia were also determined.

Haemoglobin electrophoresis was performed on vertical starch gel, according to Smithies [8] in a discontinuous buffer system tris – EDTA borate pH 8.1 – borate pH 9.0. The quantitation of each fraction was carried out according to Ricco et al [6]. For electrophoresis, fresh haemoglobin, treated with potassium cyanide immediately before use, was utilized, to avoid the formation of slow moving components which tend to migrate into the same position as normal foetal haemoglobin. Foetal haemoglobin was also determined in all the cases by the method of Betke [1]

Results

The summary of the results is illustrated in figures 1 and 2.

Normal subjects In this group the mean value of Hb A_2 has been found to be 2.32 % (±0.38) the mean value of Hb F 0.80 % (±0.94) The value of Hb F is higher than those obtained by means of alkali-denaturation according to Betke by this method the mean value has been found to be 0.59% (±0.19)

The higher values of Hb F on starch gel electrophoresis could be due to a slight contamination of Hb F by Hb A this cannot happen in the case of Hb A_2, since its separation both from the other normal haemoglobin components and from the carbonic anhydrase is very clear For this work the mean values with a tolerance of 2 σ are considered as still normal. The highest normal value of Hb A_2 is therefore 3.10 % and that of Hb F 1.30 %

Heterozygous thalassaemia. In relation to the electrophoretic picture the subjects examined can be divided into three groups the first with an isolated increase of Hb A_2, the second with a simultaneous increase of Hb A_2 and Hb F and the third with an isolated increase of Hb F We should mention that all the thalassaemic subjects examined belong to one or to another of these groups, that is, in every case a patho-

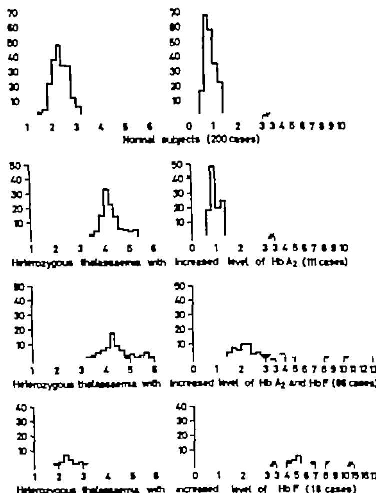


Fig 1 Distribution of Hb A_2 (left) and Hb F (right) in patients suffering from heterozygous β- and βδ-thalassaemia, compared with normal subjects. Abscissa percent of haemoglobin ordinate number of cases.

logical electrophoretic picture was present. 111 thalassaemic subjects fall into the first group the Hb A_2 values vary between 3 48 and 5 23% and those of Hb F between 0.52 and 1 30 % The second group contains 68 subjects the Hb A_2 values range between 3 42 and 5 85% Some cases in this group present very high values of Hb A_2, the highest we met in our screening The Hb F values range between 1 38 and

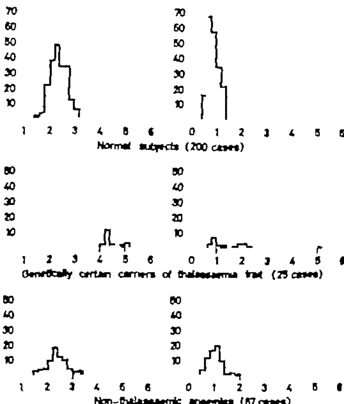


Fig. 2. Distribution of Hb A_2 (left) and Hb F (right) in healthy genetically certain carriers of thalassaemia trait and in patients suffering from non-thalassaemic anaemia, compared with normal subjects. Abscissa: percent of haemoglobin; ordinate: number of cases.

12.55%. In this group most of the cases showed relatively low values of Hb F: in only about 25% of the cases the value of Hb F is higher and comparable to those observed in the third group which presents an isolated increase of Hb F. The third group consists of 18 subjects belonging to 9 families. In these the variation of the electrophoretic pattern is due to an isolated increase of Hb F. This fraction even reaches very high values, varying between 4.04 and 16.73%, while the Hb A_2 ranges between 1.77 and 3.03%. Some subjects in this group are certainly affected by $\beta\delta$-thalassaemia, for the others we are as yet unable to give reliable data, as the family study has not yet been completed.

Genetically certain carriers of thalassaemia trait. The haemoglobin picture was found to be pathological in all the 25 carriers of thalassaemia trait. They were suspected to be such during the family investigation, but they were clinically and haematologically normal. As can be seen in figure 3 they all show a raised Hb A_2 level which ranges between 3.38 and 5.37 %. In 11 cases there is also an increased Hb F.

Subjects suffering from sickle-cell thalassaemia. In these subjects we observed two different electrophoretic patterns (fig. 3). The most common (3 cases) is characterized by Hb A (11–14 %), Hb S (74–84 %), Hb F (5–11 %) and Hb A_2 (3.5–4.5 %). The second pattern (1 case) shows a normal level of Hb A_2 (2.87 %), a high level of Hb F (9.4 %) and Hb S (87.73 %). Hb A was absent.

Anaemias of different types. The haemoglobin picture was found to be normal in 64 out of 67 patients. The Hb A_2 level in 3 cases was a little higher than our normal value: these patients belonged to non thalassaemic families and their anaemia was not microcytic. In 4 cases we have also found a slight isolated increase in Hb F.

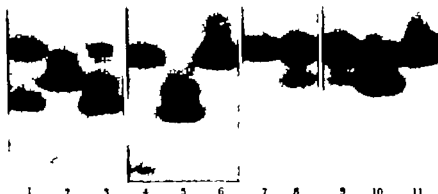


Fig. 3. Starch gel electrophoresis tris-EDTA-borate pH 8.1 of: (1) A-S heterozygote; (2) Cooley's anaemia with small amount of Hb A and high Hb A_2; (3) sickle-cell thalassaemia: some Hb A is present, Hb A_2 and Hb F are consistently increased; (4) heterozygous β-thalassaemia: Hb A_2 is raised, small amount of Hb F is present; (5) sickle-cell thalassaemia: in this case Hb A is absent, Hb F is highly increased and Hb A_2 is in the normal limits; (6) heterozygous β-thalassaemia with an isolated increase of Hb A_2; (7) normal subject; (8) heterozygous β-thalassaemia with simultaneous increase of Hb A_2 and Hb F; (9) βδ-heterozygous thalassaemia: Hb A_2 is in the normal limits, while Hb F is clearly raised; (10) Cooley's anaemia: Hb A_2 increased and Hb A is nearly absent; (11) non-thalassaemic anaemia.

Discussion

The results obtained in healthy subjects are similar to those described by other authors and confirm that these values range within narrow limits. In all the cases of heterozygous β- and βδ-thalassaemia one or more than one fraction showed a statistically significant variation. In 57% of our patients we observed an isolated increase in Hb A_2 in 34% a simultaneous increase in Hb A_2 and Hb F and in 9% an isolated increase in Hb F.

The first two patterns, according to WEATHERALL [10] are typical for the heterozygous β-thalassaemia the difference between these two pictures, however does not exclude the possibility that they may be two distinct forms of thalassaemia [7]. We shall return to this subject when we have studied a sufficiently large number of families. However we can say that the haemoglobin picture does not seem to have any relation with the seriousness of the clinical picture and the degree of anaemia. The isolated increase of Hb F is characteristic of βδ-thalassaemia however an isolated increase in Hb F has been found in members of families with typical β-thalassaemia.

Apart from any genetical interpretation which might be formulated on the bases of our results, we must point out that all the thalassaemic subjects examined, showed characteristic alterations of their haemoglobin picture. These results seem to point to the fact that the quantitation of the haemoglobin fractions obtained by starch gel electrophoresis is enough, by itself, to recognize with certainty all the cases of heterozygous β- and βδ-thalassaemia. The study concerning the carriers of non thalassaemic anaemias confirms the validity of the method in fact only in 4.5% of the cases an abnormal electrophoretic picture, characterized by a modest rise of Hb A_2 was obtained these patients were certainly not suffering from thalassaemia, and affected by normocromic anaemia. The modest rise in Hb F observed in some cases does not allow us to make a diagnosis of thalassaemia, and the thalassaemic subjects presenting an isolated increase on Hb F show values not inferior to 4 For this condition WEATHERALL gives values of 5 or more.

Quantitative starch gel electrophoresis allows us to detect only some cases of double heterozygotes for the β-thalassaemia gene and for a gene for an abnormal β-chain variant of Hb A. In these cases diagnosis is possible when Hb A_2 is raised in all other cases only a

family study can provide a sure diagnosis. In cases of double heterozygotes for β-thalassaemia gene and for a gene for an abnormal α-chain variant of Hb A, the diagnosis can be easier but we must point out that a rise in Hb A_2 can be hidden as the Hb A_2 fraction carrying the α-chain abnormality has an abnormal mobility On the other hand, we have observed that the method is quite useless for the diagnosis of heterozygous carriers of α-thalassaemia I and β-thalassaemia II unless haemoglobin H or haemoglobin Bart's is present.

Nevertheless, our results on the whole seem to indicate that the quantitation of haemoglobin fractions obtained by starch gel electrophoresis is a sufficiently foolproof method in the screening of the thalassaemia syndromes.

Summary

The authors have analysed on starch gel electrophoresis 195 heterozygotes for β- and $\beta\delta$-thalassaemia with clinical and haematological abnormalities, 25 certain carriers of the thalassaemia gene, without any pathological findings and 4 patients suffering from sickle cell-thalassaemia. The results have been compared with those obtained from 200 normal subjects and from 67 patients with various non thalassaemic anaemias. In all the thalassaemic subjects an abnormal haemoglobin pattern was observed. Only 4.5% of the non thalassaemic anaemias showed slight increase of Hb A_2 and in 4 cases out of 67 the level of Hb F was higher than normal.

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Authors' address Drs. G. Ricco, E. Gallo, U Mazza, G. Bianco and V Prato, Istituto di Patologia Speciale Medica, Via Genova 3, *10126 Torino* (Italy)

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Effect of Lysolecithin on Bovine Erythrocyte Osmotic Fragility[1]

S. R. Garfin, M. A. Shea and E. F. Bernstein

Department of Surgery, University of Minnesota Medical School, Minneapolis, Minn. and University of California, San Diego

A consistent decrease in erythrocyte osmotic fragility was observed by Shea [1, 2] during studies concerning the effects of *in vitro* blood pumping and subsequent reinfusion into the donor animal. In these canine experiments, and other experiments involving 24 and 48-hour partial arteriovenous bypass in dogs [3], the erythrocyte osmotic fragility gradually decreased during the perfusions and over the five days following the experiments, and returned to normal over the subsequent 14 days. Reported observations of experimental or clinical conditions involving a decrease in osmotic fragility are not common, but one substance reported to cause such an effect is lysolecithin [4, 5]. Collier and Ponder have described an increased resistance to hypotonic hemolysis following the addition of red blood cells to sublytic concentrations of lysolecithin. However, to our knowledge, the data documenting these observations has not been published. The present experiment was designed to quantitate the effect of varying sublytic concentrations of lysolecithin on the osmotic fragility of bovine erythrocytes.

Method

Blood was drawn under sterile conditions in ACD solution from fasted calves on the day of the experiment. After centrifugation for 20 min at 2,500 rpm the plasma was separated from the red blood cells.

A stock solution of lysolecithin was prepared from commercially available material obtained from cobra venom (Mann Research Laboratories, Inc., New York, N.Y.) by adding 20 mg of crystalline lysolecithin to 10 ml saline. On the day of the experiment the solution

[1] Supported by Research grant HE-06961, National Heart Institute, USPHS.

was sonified to increase the effective solubility of lysolecithin. Stock lysolecithin solution was added to saline to obtain final concentrations ranging from 0 to 5.1×10^{-11} μM lysolecithin/RBC prior to the addition of red blood cells. Erythrocytes were added to obtain final hematocrit between 30 and 40%, gently mixed, and incubated for 30 min at 37° C. Following incubation, the osmotic fragility of the erythrocytes was determined by exposing them to progressively decreasing concentrations of sodium chloride solutions [6]. RBC osmotic fragility was also determined with the Fragiligraph Osmotic Test Recorder Model D2 (Kalmedic Instruments, Inc., New York, N.Y.) measuring the time for 50% hemolysis. The Fragiligraph was maintained at constant temperature of 37° C by Haake Model F water bath, and its output was recorded on Texas Servo-riter II potentiometric recorder. The hematocrit and plasma hemoglobin concentrations of the incubated solution were determined by the methods of WINTROBE [7] and FLINK and WATSON [8], respectively.

Results

The osmotic fragility of the erythrocytes exposed to concentrations of lysolecithin less than 1.68×10^{-11} μmoles lysolecithin/RBC did not

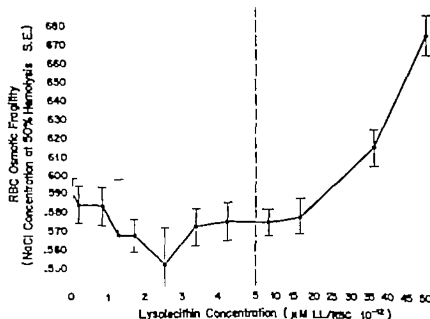


Fig. 1. Plot of RBC osmotic fragility (determined by manual method) versus lysolecithin concentration, with standard error of the mean indicated for each concentration. A modest and statistically insignificant decrease in osmotic fragility is noted at lysolecithin concentrations in the range of 2.5 $\times 10^{-12}$ μM lysolecithin/RBC. Significant changes, however, are not seen until concentrations exceeding 20 $\times 10^{-12}$ μM lysolecithin/RBC were examined and these are in the direction of increasing fragility.

demonstrate any significant change from the controls (fig 1) There was no consistent *decrease* in osmotic fragility in any of the 12 experiments. At higher concentrations of lysolecithin, ranging from 1 68 to 5 08 × 10^{-11} μM lysolecithin/RBC, there was a gradual INCREASE in osmotic fragility Duplicate measurements performed in the Danon Fragiligraph (fig 2) demonstrate similar changes, but the magnitude of the changes and statistical significance of the data are not as great.

Discussion

In continuing studies concerning the mechanism of mechanically induced hemolysis, our laboratory has focused its attention on several of the late effects observed following the exposure of canine, human and bovine blood to various forms of mechanical stress. One of these effects

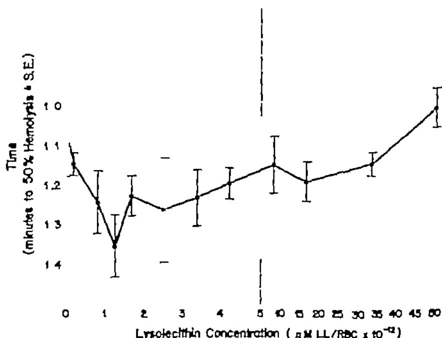


Fig. 2. Erythrocyte osmotic fragility as determined by the time for 50% hemolysis in the Danon Fragiligraph, is plotted against lysolecithin concentration. There appears to be slight decrease in fragility at low concentrations, but this is statistically insignificant. At higher concentrations, again, an increase in osmotic fragility was observed.

has been the peculiar and consistent finding of an increase in resistance to osmotic stress, or decrease in osmotic fragility after approximately ¼ of the blood volume of dogs was pumped *in vitro* and then reinfused into the donor animal [1] Of interest is the additional observation that when only a fraction of the animal's cells have been exposed to trauma, not only these, but all of the animal's cells, subsequently demonstrate alterations in osmotic fragility [1] Additional experiments failed to demonstrate any consistent changes in erythrocyte volume which might help explain these observations [2]

One substance which has been suggested as participating in both the normal lysis of aged red cells and in the damage observed following exposure to artificial surfaces is lysolecithin. This substance has been reported to cause a decrease in erythrocyte osmotic fragility in sublytic concentrations [4 5] Inasmuch as the literature concerning this aspect of the effects of lysolecithin appeared scanty an appropriate experiment to evaluate the osmotic effects of lysolecithin in sublytic concentrations was performed.

In observing the effects of similar concentrations of lysolecithin upon rabbit RBC morphology WATTERS[1] has observed an initial stage of crenation at lysolecithin concentrations of 5×10^{-11} μM LL/RBC, followed by sphering at lysolecithin concentrations approximating 2×10^{-11} μM LL/RBC, and finally by lysis at concentrations greater than 5×10^{-1} μM LL/RBC. This sequence can be reversed by the addition of bovine serum albumin prior to hemolysis. Albumin is known to actively bind lysolecithin. It therefore appeared logical that, at the stage of crenation, cells exposed to appropriate concentrations of lysolecithin might well have an increased resistance to osmotic stress. That such results were not observed casts doubt upon the role of lysolecithin as a significant participant in the effects of mechanically induced hemolysis. Furthermore, experiments directed at measuring the release or appearance of significant amounts of lysolecithin during or following the exposure of bovine, canine, human and mouse blood to mechanical stress have failed to demonstrate an increase in the concentration of lysolecithin in either red blood cells or plasma[2] These observations, when coupled with the results of the present experiment, make the proposed lysolecithin hemolytic mechanism unlikely

[1] Unpublished data.
[2] BERNSTEIN, E. F unpublished data.

demonstrate any significant change from the controls (fig 1). There was no consistent *decrease* in osmotic fragility in any of the 12 experiments. At higher concentrations of lysolecithin ranging from 1.68 to 5.08×10^{-11} μM lysolecithin/RBC, there was a gradual INCREASE in osmotic fragility. Duplicate measurements performed in the Danon Fragiligraph (fig 2) demonstrate similar changes, but the magnitude of the changes and statistical significance of the data are not as great.

Discussion

In continuing studies concerning the mechanism of mechanically-induced hemolysis, our laboratory has focused its attention on several of the late effects observed following the exposure of canine, human and bovine blood to various forms of mechanical stress. One of these effects

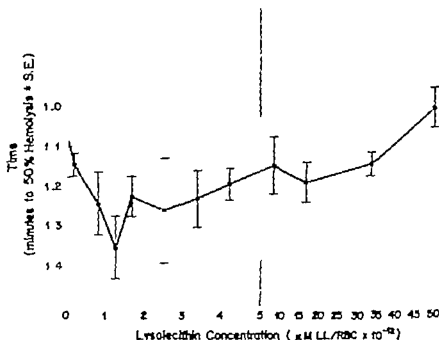


Fig 2. Erythrocyte osmotic fragility, as determined by the time for 50% hemolysis in the Danon Fragiligraph, is plotted against lysolecithin concentration. There appears to be slight decrease in fragility at low concentrations, but this is statistically insignificant. At higher concentrations, again, an increase in osmotic fragility was observed.

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Department of Chemical Pathology, Government Hospital and Tel-Aviv University Medical School, Tel-Hashomer and Department of Pediatrics, Government Hospital, Hadera

Hereditary Deficiency of Adenylate Kinase in Red Blood Cells

A. Szeinberg, D. Kahana, Slava Gavendo[1], J. Zaidman and Judith Ben Ezzer

Adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) catalyses the interconversion of adenine nucleotides according to the following equation: 2 ADP ⇄ ATP+AMP. High activity of this enzyme has been found in red blood cells and in some other tissues [1]. In 1966 Fildes and Harris [2] described a genetic polymorphism of this enzyme in human red cells. Several accounts on relative frequency of the genes governing this polymorphism in different populations have been recently published [3–8].

Within the frame of an investigation of this genetic trait in different population groups in Israel a blood sample demonstrating a deficiency of adenylate kinase (AK) in red cells was encountered. Family investigations demonstrated a hereditary transmission of this deficiency. In the same family an additional genetic defect has been detected, namely glucose-6-phosphate dehydrogenase (G6PD) deficiency due to an electrophoretically fast mutant. These investigations are described in the present report. A preliminary report has been published [9].

Methods

Preparation of hemolysates. Heparinized blood was centrifuged for 5 min at 1,200 *g* in a refrigerated centrifuge (4°C). Plasma and buffy coat were removed. The red cells were washed three times in cold saline and centrifuged each time for 5 min at 3,000 *g*. Hemolysates for starch gel electrophoresis for adenylate kinase and phosphoglucomutase (PGM) were prepared by freezing and thawing in mixture of dry-ice acetone. Hemolysates for starch gel electrophoresis for G6PD were prepared according to the recommendations of WHO Scientific Group [10].

[1] Work performed in partial requirement for M.Sc. degree at the Department of Microbiology, Tel-Aviv University.

Hemolysates for quantitative enzyme assays were prepared by lysis of packed red cells in 3 volumes of distilled water. All the examinations were carried out on stroma free hemolysates prepared by centrifuging at 41,000 *g* for 20 min at 4 C.

Starch gel electrophoresis. Examination for AK was carried out by the horizontal method of FILDES and HARRIS [2], in cold room (4 C), but at 5.5 V/cm for 17 h. PGM was examined by the horizontal method of SPENCER *et al.* [11], in cold room (4 C) but at 6.5 V/cm for 24 h. The staining mixture was prepared according to MOORE [12].

Starch gel electrophoresis for G6PD was performed in the following systems: tris chloride buffer [10], EDTA-boric acid-tris [10] and phosphate buffer [13]. The staining was carried out with solutions containing MTT tetrazolium and phenazine methosulphate [10] or nitro-blue tetrazolium and methylene blue [14].

Quantitative Enzymatic Assays

Adenylate kinase. AK activity in the forward direction was determined by measuring the production of ATP with the aid of auxiliary enzymatic reactions involving hexokinase (HK) and G6PD [15] in a system coupled with the reduction of NADP to NADPH. The assay mixture (3 ml) contained the following final concentrations of reagents: 0.1 M tris HCl buffer pH 8.0, 10 mM glucose, 1 mM ADP, 0.4 mM NADP, 20 mM $MgCl_2$, 0.12 U/3 ml G6PD, 0.24 U/3 ml HK, 0.01 ml/3 ml hemolysate. The reduction of NADP was followed at 340 nm at 26°C in Zeiss Spectrophotometer. Before the addition of the hemolysate there was a rise of extinction during a few minutes probably due to residual traces of ATP in the ADP. When no more change in optical density was observed, 0.01 ml of hemolysate containing 1.5–2.5 g% Hb was added. There was usually a lag period of about 5 min before the reaction became linear with time. The enzymatic activity was calculated from the rate of reaction observed after the lag period.

AK activity in the backward reaction was determined by measuring the production of ADP with the aid of auxiliary reactions involving pyruvate kinase (PK) and lactic dehydrogenase (LDH) in a system coupled with the oxidation of NADH to NAD [15]. The assay mixture (3 ml) contained the following final concentrations of reagents: 0.073 M triethanolamine-HCl (TRA) buffer pH 7.6, 1.33 mM AMP, 1.1 mM ATP, 0.36 mM phosphoenol pyruvate, 0.52 mM $MgSO_4$, 133 mM KCl, 0.25 mM NADH, 3 U/3 ml PK, 36 U/3 ml LDH, hemolysate (1.5–2.5 g% Hb) 0.01 ml/3 ml. The assay mixture with hemolysate but without ATP did not show any change in optical density. On the other hand the mixture without AMP but with ATP produced a gradual decrease of extinction at 340 nm lasting for several minutes. Consequently all the assays were started without AMP which was added only after stabilization of optical density. Also in this reaction there was an initial lag period of several minutes and the calculations were based on activity observed after this period.

The reproducibility of adenylate kinase assays in blood samples from normal subjects examined in duplicates simultaneously or on consecutive days was within 10% range.

Aldolase was assayed in a system coupled with glycerol-1-phosphate dehydrogenase (GDH) and triosephosphate isomerase (TIM) [16]. The assay mixture (3 ml) contained the following final concentrations of reagents: 0.045 M TRA buffer pH 7.5 containing 2.6×10^{-4} M iodoacetate, 0.23 mM NADH, 0.75 U/3 ml GDH, 4.5 U/3 ml TIM and 0.1 ml/3 ml hemolysate (about 4 g% Hb). The reaction was started with fructose 1,6 diphosphate (4.0 mM final concentration).

Glucose-6-phosphate dehydrogenase was assayed by the slightly modified [17] method of KORNBERG and HORECKER [18].

Glutathione reductase [19]. The assay mixture (3 ml) contained the following final concentrations of reagents: 0.043 M tris-HCl buffer pH 7.6, 33 mM EDTA, 0.15 mM NADPH, 0.03 ml/3 ml hemolysate (about 4 g% hemoglobin). The reaction was started with oxidized glutathione (GSSG) (0.22 mM final concentration).

Hexokinase was assayed in system coupled with G6PD [20]. The assay mixture (3 ml) contained the following final concentrations of reagents: TRA EDTA buffer (triethanolamine 0.025 M, pH 7.4 and EDTA 0.1%) 2 mM NaF 8 mM $MgCl_2$, 2 mM glucose, 0.08 mM NADP 0.024 U/3 ml G6PD, and 0.1 ml/3 ml hemolysate (about 4 g% Hb). The reaction was started with ATP (1.5 mM final concentration).

Lactic dehydrogenase [21]. The assay mixture (3 ml) contained the following final concentrations of reagents 0.05 M phosphate buffer pH 7.5, 0.31 mM sodium pyruvate, 0.002 ml/3 ml hemolysate (about 4 g% Hb). The reaction was started by addition of NADH (0.15 mM final concentration).

Pyruvate kinase [22] was assayed in system coupled with LDH. The assay mixture (3 ml) contained the following final concentrations of reagents TRA-HCl buffer 0.030 M pH 7.5, 1.5 mM phosphoenolpyruvate 12 mM $MgSO_4$, 110 mM KCl, 0.15 mM NADH, 18 U/3 ml LDH, 0.030 ml/3 ml hemolysate (about 4 g% Hb). The reaction was started by the addition of ADP (3.4 mM final concentration).

The auxiliary enzymes, coenzymes and substrates used in the above spectrophotometric assays were purchased from Boehringer Co., Mannheim. The results of the enzyme activities were expressed as µmoles/min/g Hb at 26°C.

Catalase was determined by the method of FEINSTEIN [23] as modified by TARLOV and KELLERMEYER [24] with incubation time of 1 min.

Adenine nucleotides. ATP ADP and AMP were measured enzymatically with appropriate assay kits of Boehringer Co., Mannheim (TC-J No. 15979 TAAC and TC-K, No. 15980 TAAB). The determinations were carried out on whole blood heparinized and deproteinized with cold perchloric acid immediately after removal from the vein. The results were calculated per 100 ml of packed cells, representing therefore the content of all the cellular elements of blood, and not of red cells only.

Osmotic fragility autohemolysis (24 h at 37°C) and Heinz bodies were examined according to DACIE [25].



Fig. 1. Adenylate kinase in hemolysates. Starch gel electrophoresis examined by the method of FILDES and HARRIS [2]. 2 = propositus, 1 = normal subject, AK phenotype 1. 3 and 4 = normal subjects, AK phenotype 2-1. The lowest spot in each sample is hemoglobin.

Case History

During an investigation of genetic polymorphism of AK among various populations groups in Israel, one blood sample showed an almost complete absence of AK activity on examination by starch gel electrophoresis (fig 1) The main isozyme components of AK were practically missing and only one minor anodic component was observed on the electropherograms. The mobility of this fraction was slightly different from those of the two minor components observed in normal blood controls. The sample was obtained from an Arab boy aged 5 months with the following family history (table I): his older brother was hospitalized several times in 3 hospitals since the age of 2 years. His hemoglobin values fluctuated between 3.7 and 8.6 g%, erythrocyte counts between 1,200,000 and 2,620,000, the reticulocytes between 2.9 and 34%. Some degree of hyperbilirubinemia was always present (2.3–9.4 mg%) No abnormal hemoglobins were found. The half life of erythrocytes measured with ^{51}Cr was considerably shortened. He was found to have G6PD deficiency but the enzyme values fluctuated between 0 and 1.8 U/g Hb (normal values 4.0–7.2) The condition of the boy was diagnosed as non spherocytic hemolytic anemia with G6PD deficiency The boy died at home at the age of 7 years with cause of death unknown and before any studies on the type of G6PD deficiency could be carried out.

In view of this family history when the propositus was born, the parents were advised to bring him for examination at the age of 2 weeks. It was found then that he had hepatomegaly severe anemia (6.6 g% Hb) slight jaundice and no detectable activity of G6PD in red cells. He was given a blood transfusion. Four months later again severe anemia with reticulocytosis of 5.5% and normal serum iron was observed. Physical examinations demonstrated hepato-and splenomegaly A blood sample obtained at that time showed an absence of G6PD and revealed the deficiency of AK on starch gel electrophoresis. One day after this blood sample was collected the child received again a blood transfusion and further investigations were postponed for several months.

Table I Summary of observations on the propositus (up to the time of diagnosis of adenylate kinase deficiency) and his deceased brother

	Propositus age 2 weeks	Propositus age 5 months	Brother (deceased) examinations at ages 2 7 years
Hb, g%	6.6	5.1 5.6	3.7–8.6
Hematocrit, %			13–30
RBC, 10⁶	2.50		1.20–2.62
Reticulocytes, %		5.5	2.9–34
RBC survival (^{51}Cr)			half life 9 days
Serum bilirubin, mg%	3.3		2.3–9.4
Serum iron, µg%		120	45–140
Fecal stercobilin			24–130 mg/24 h
G6PD, U/g Hb	0	0	0–1.8
Adenylate kinase		deficient by electrophoresis	not tested
Clinical notes	hepatomegaly blood transfusion	hepatosplenomegaly blood transfusion	hepatosplenomegaly multiple blood transfusions

Results

AK and G6PD were determined in red cells of both parents, the propositus (120 and 160 days after the last transfusion) and his 7 living sibs. The same samples were examined also by starch gel electrophoresis for determination of AK and PGM phenotypes. The results are summarized in table II.

AK activity. Examinations of the blood of the propositus obtained on several occasions suggested some variability in the degree of the deficiency of this enzyme. As previously mentioned, the blood taken at the age of 5 months demonstrated only one minor band of activity on starch gel electrophoresis. A second blood sample obtained at the age of 9 months (120 days after the last transfusion) showed in quantitative assays about 10 of mean normal AK activity and on starch gel electrophoresis a faint pattern corresponding to the phenotype AK 1. In another blood sample obtained 40 days later about 4% of mean normal activity was present and on starch gel electrophoresis again only one minor band was seen. The family investigation revealed that a sister of the propositus (F I V/3) aged 4 years, also showed an almost complete absence of AK activity. In quantitative assays 0.5–4% of mean normal AK values were found and on the starch gel electrophoresis only the minor component was observed. All the other members of the family showed a partial deficiency of AK. The enzyme activities in their red cells measured in the 2 directions were below 2 standard deviations from the means of control groups. However considerable variation in expression was observed and the enzyme activity in various members ranged between 22 and 72% of mean normal values. We interpret the above data as being compatible with a single factor autosomal inheritance of AK deficiency with a variable expressivity in heterozygotes.

Additional investigations of AK. The hemolysate prepared from red cells of the AK deficient girl did not show any inhibitory activity upon addition to normal hemolysates in the assay mixture for AK. Some experiments were performed to prove that the minor band of activity seen on electropherograms of the enzyme deficient subjects was indeed due to AK and not to secondary reactions. The colour development of this band did not require more time than the specific pattern of AK in normal subjects. Omission of glucose, ADP, hexokinase or G6PD prevented the staining of this minor component.

Table II. Family investigation: enzyme data

Subject	Relation to propositus	Age years	AK activity forward direction absolute values	forward % of mean normal value	AK activity backward direction absolute values	backward % of mean normal value	G6PD activity	AK phenotype	PGM phenotype	Blood group
IV/1 I.L.I.	father	38	44.2–53.3	49.3–59.4	66.6–78.5	58.6–69.0	5.0–5.7	1	2-1	B, Rh pos.
IV/2 B.I.	mother	32	25.6–31.1	28.5–34.7	25.8–35.4	22.3–31.1	1.4–3.0	1	1	AB, Rh pos.
V/1 R.I.	propositus	11/12	9.4	10.5	4.4–8.8	3.8–7.6	0–1.6	1	2-1	B, Rh pos.
V/2 A.I.	sister	2	41.4	46.2	42.4	37.3	3.2	1	1	AB, Rh pos.
V/3 T.J.	sister	4	0.5–1.3	0.56–1.4	1.9–4.7	1.7–4.2	3.0	?	2-1	
V/4 M.I.	sister	5	64.3	71.7	64.3	56.6	5.0	1	2-1	AB, Rh pos.
V/5 N.I.	sister	7	53.8	60.0	64.9	57.1	4.7	1	1	
V/7 G.I.	brother	10			82.0	72.1	3.0	1	2-1	
V/8 Z.I.	brother	12	64.9	72.4	79.3	69.9	4.4	1	1	B, Rh pos.
V/9 N.I.	brother	14	61.0	68.0	76.8	67.5	4.0	1	1	
Normal range			73.4–106.1		97.9–132.3		4.0–7.2			
Normal mean ± SD			89.7 ± 10.0		113.7 ± 10.6					

Number according to the family tree (fig. 3).

Expressed in µmoles/min/g Hb at 25°C. When a range of activities is given, the figures represent results obtained on examination of several samples at some months intervals; in other cases the figures represent mean values of duplicate examinations of one blood sample.

12 subjects examined.

22 subjects examined.

G6PD activity The first 2 samples obtained from the propositus at the age of 2 weeks and 5 months did not show any G6PD activity while in samples obtained at the age of 9 months (120 days after transfusion) and 10 months (160 days after transfusion) activities of 1 6 and 0.9 units respectively were observed. These fluctuations will be discussed later but for the sake of description of the phenotype the case will be regarded as severe G6PD deficiency

Crude hemolysates prepared from blood samples obtained at the ages of 9 and 10 months were examined by starch gel electrophoresis In the three buffer systems used (see Methods) the mobility of G6PD of the propositus was more rapid than the normal G6PD type B (fig 2) This observation suggested that the G6PD deficiency of the propositus was not of the usual Mediterranean type prevalent in our population.

Examination of the family demonstrated intermediate G6PD deficiency in the red cells of the mother and one of the sisters of the propositus (A.I V/2) The father of the family the sister with AK deficiency and 6 other sibs of the propositus showed normal G6PD

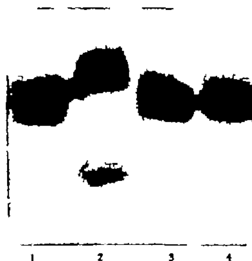


Fig 2. G6PD in hemolysates. Starch gel electrophoresis in tris-EDTA buffer [10]. 1 and 4 = normal subjects, G6PD type B 2 = propositus 3 = adenylate kinase deficient sister of the propositus (F I V/3) The origin is the bottom of the figure. The lower bands represent hemoglobin and the upper bands G6PD. The hemolysate of the propositus contained 3.2 g% hemoglobin, the other hemolysates 1 6 g% hemoglobin. A similar separation but much fainter bands were obtained with hemolysates containing 0.4 g% (controls) and 0.8 g% (propositus) hemoglobin.

Table III Hematologic data obtained at the time of enzyme studies in the patient and his family

		Propositus (age 9–11 months)	Sister (F.I V/3)	Father (H.I IV/1)	Mother (S.I IV/2)	Normal range
Hb, g%		6.5–7.1	8.5–9.0	14.2	10.7 11.0	
RBC, 10^6		2.6–2.67	2.97 3.40	5.10	3.71 –3.78	
Hematocrit, %		25–27	27–29	46	34–38	
Reticulocytes, %		8.4–12	2.0–3.2	0.5	0.3–0.8	
Serum bilirubin, mg% (total/direct)		1.9/0.1 2.4/0.5	1.4/0.3–1.7/0.5	–	0.9/0.5	
Serum iron, µg %		141	120	–	50	
Osmotic fragility		normal	normal	normal	normal	
Autohemolysis, %/24 h/37°C	without glucose	3.6	2.1	0.9	1.3	0.2 1.0
	with glucose	5.9	0.7	0.6	0.5	0.1–0.8
MCV µm³		93.6–103.8	85.3–90.9	90.2	91.6–101.9	76–96
MCH, pg		24.3–27.3	26.5–28.6	27.8	28.8–29.1	27–32
MCHC, %		25.8–26.0	31.0–31.5	30.9	28.6–31.5	32–36
Blood film		macrocytes anisocytosis poikilocytosis polychromia occasional normoblasts	few macrocytes slight anisocytosis	normal	normal	

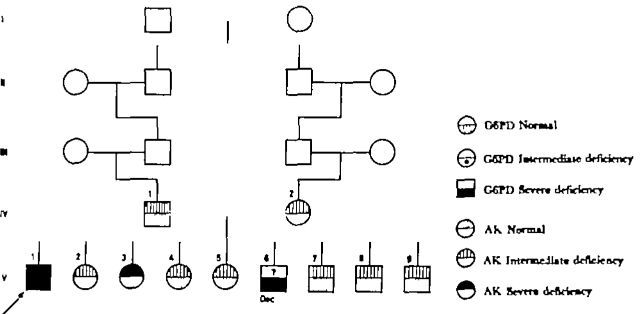


Fig 3. Family tree.

activity The family observations are summarized in figure 3 demonstrating that the propositus had a severe deficiency of both AK and G6PD his sister (F I V/3) severe deficiency of AK alone, 2 members of the family (S I IV/2 and A.I V/2) partial deficiency of the two enzymes, while the remaining members of the family partial deficiency of AK alone.

Hematologic observations (table III) The blood examinations of the propositus performed at the age of 9–11 months showed a hypochromic anemia (Hb 6.5–7 1 g%) with marked reticulocytosis (8 4–12 %) and moderate hyperbilirubinemia. The serum iron and osmotic fragility were normal, while autohemolysis was increased (5 6%) and not corrected by glucose. These results suggested that most probably the child suffered from chronic non spherocytic hemolytic anemia.

The sister (F I V/3) which had severe AK deficiency but normal G6PD also showed an anemia (normocytic, normochromic) though in somewhat milder degree. Her Hb was about 9 g % and RBC about 3 000 000 She had only slight reticulocytosis (2–3.2 %) and slight hyperbilirubinemia (1 4–1 7 mg %) Serum iron and osmotic fragility were normal. Autohemolysis in the absence of glucose was slightly above the normal range observed by us in control subjects. It is of interest to note that the finding of anemia in this child was rather unexpected. The girl (4 years old) was not considered by the parents to be ill and as far as it is known to us she was never brought for medical examination When the anemia has been detected in the present investigation, physical examination revealed a pale and apathetic child without enlargement of liver or spleen and without any other particular findings

A mild degree of anemia without reticulocytosis or hyperbilirubinemia was also observed in the mother with partial deficiency of G6PD and AK, while the hematologic findings in the father were normal. The WBC and platelets counts were normal in both parents and the two AK deficient children. Heinz bodies were not found in any of these subjects.

Activity of other enzymes in red cells (table IV) The activity of several enzymes has been measured in the blood of these 4 members of the family The propositus showed markedly increased activity of pyruvate kinase, aldolase glutathione reductase and hexokinase, reflecting most probably the presence of a high proportion of young cell population Increased activity of hexokinase and glutathione reductase was also

Table IV Activity of some red cell enzymes in the AK deficient children and their parents

Enzyme	Propositus	Sister (P.I V/3)	Father (H.I IV/1)	Mother (S.I IV/1)	Control values Normal subjects = 14	G6PD def. subjects = 10
Aldolase	*3.85*	2.62	2.26	2.98	2.96 ± 0.38	3.71 ± 0.46
Glutathione reductase	*7.1*	*4.1*	3.8	*3.57*	3.27 ± 0.28	5.52 ± 0.81
Hexokinase	*0.57*	*0.67*	0.37	0.30	0.31 ± 0.03	0.46 ± 0.01
Lactic acid dehydrogenase	125.0	101.0	108.0	96.0	102.0 ± 11.0	106.0 ± 10.10
Pyruvate kinase	*17.8*	11.2	9.8	11.0	9.7 ± 0.77	10.7 ± 1.20
Catalase	0.25	0.24	0.26	0.22	0.2 ± 0.035	0.19 ± 0.030

Catalase activity in [illegible] units; other enzymes expressed as µmoles/min/g Hb.
Data in italics denote activity above 2 standard deviations from the mean of control normal subjects.

Table V Adenine nucleotides content of blood cells (µmoles/100 ml cells)

	ATP	ADP	AMP	Percent ADP*
Propositus	151 4	39.8	7.6	20.0
Sister (F I V/3)	141.0	38.9	6.9	20.5
Father (H.J IV/1)	111.0	24.8	3.4	17.8
Mother (S.J IV/2)	113.8	31.0	4.4	20.8
Controls with reticulocytosis				
Case 1 (retic. 13%)	180.3	29.2	5.8	13.6
Case 2 (retic. 3.5%)	126.8	18.0	2.2	12.2
Case 3 (retic. 2.6%)	147 1	14.3	6.9	8.6
Normal range (= 16)	88.5–137.0	14 7 24.8	1.2 5.2	10.0–18.9
Normal mean ± SD	108.4±13.6	17.9±2.9	3.3±1.0	14.0±2.8

Percent ADP was calculated by the formula $\frac{ADP \times 100}{ATP + ADP + AMP}$

observed in the red cells of the sister with severe AK deficiency while the mother showed an increased activity of glutathione reductase only

Adenine nucleotides content of blood (table V) In comparison with normal subjects elevated concentrations of ATP ADP and AMP were observed in the cells of the propositus and his AK deficient sister However the relative increase of the ADP seemed to be higher than that of ATP and AMP In both AK deficient subjects and their mother ADP constituted about 20% of the sum of adenine nucleotides, while the mean value observed in normal controls was 14 0±2 8% In one control subject with marked reticulocytosis and 2 subjects with mild reticulocytosis the relative content of ADP was only 8 6–13 6%

Discussion

In this family two genetic enzymatic defects of red cells have been detected, namely a deficiency of AK and a deficiency of G6PD due to a non-Mediterranean variant. The genes for those two abnormalities segregated independently and different combinations have been observed in various members of the family The results suggested that the AK deficiency was transmitted by an autosomal gene with a partial and variable expressivity in heterozygotes. The G6PD deficiency was

not due to the Mediterranean variant prevalent in our area, as a mutant enzyme with an electrophoretic mobility faster than G6PD type B was found in the red cells of the propositus (the parents were not yet examined in this respect) No detailed characterization studies of the mutant enzyme have yet been performed and thus its identity remains to be determined. The most common variant with increased electrophoretic mobility is the A– (African) type leading to drug sensitive hemolysis. Other fast variants have been described in cases with or without non spherocytic hemolytic anemia [10 26, 27]

In the red cells of the propositus marked fluctuations of both G6PD and AK activity were observed in samples obtained at different times. Their cause is at present not clear It does not seem probable that they were due to remnants of transfused blood, as the samples were obtained always at least 120 days after transfusion. The fluctuations did not show a correlation with reticulocyte counts, and thus could not be explained on the basis of changes in relative proportions of a younger cell population.

The nature of the metabolic burden imposed by the AK deficiency has not yet been elucidated. The physiological function of this enzyme is not exactly known. Harris et al [28] suggested that it probably plays a specific role in maintaining cellular ATP content. According to Mills et al [29] AK is essential for the conversion of AMP to ADP Several other investigators demonstrated that AK is efficient in regulating the equilibrium of adenine nucleotides in tissues even when the energy charge varies rapidly and thus it plays an important role in regulation of the energy metabolism and of the activity of many enzymatic systems [30 31 32 33] We thought that the analysis of adenine nucleotide content of blood of AK deficient subjects would throw some light on the metabolic importance of this enzyme and the direction in which the equilibrium represented by AK reactions is favored in the body We presumed that a metabolic block due to the enzyme deficiency would result in accumulation of the products at one side of the equilibrium with a deficiency at the other side. However the results of the analysis of the 2 AK deficient subjects did not bear out this expectation. All the three adenine nucleotides were found in increased concentrations. The only clue to the possible effect of enzymatic abnormality was the finding that the relative content of ADP was higher in the enzyme deficient cells than in most normal controls or control subjects with slight or moderate reticulocytosis. In recently

published observations of Mills *et al.* [29] elevated levels of ATP and AMP were found in cases with young cell population. However they were not accompanied by a parallel increase of the ADP levels. The mean relative ADP content of red cells of normal subjects studied by these investigators was 12.4%, while in a heterogenous group of subjects with a younger cell population it was 11.1%. Our finding of a relatively increased ADP content of the AK deficient cells suggests a possibility that in normal red cells the enzyme acts mainly in the direction of the conversion of ADP to ATP and AMP. However the data are limited and additional investigations are required before drawing definite conclusions.

The hematological data obtained in the girl with AK deficiency suggest the presence of a mild non-spherocytic hemolytic anemia, conforming to the type 1 of Selwyn and Dacie [34]. On several examinations a moderate anemia has been observed with normal serum iron and osmotic fragility, slightly increased autohemolysis correctable by glucose, slight hyperbilirubinemia and slight reticulocytosis. However obvious relationship between the enzyme deficiency and the anemia has not been established with certainty. The investigation of this case has been hampered by the fact, that the family was quite unco-operative. The parents do not consider the girl to be ill and they refuse to give consent to hospitalization or any investigative procedures beyond the donation of a few samples of venous blood.

The propositus showed a severe anemia with pronounced reticulocytosis and hyperbilirubinemia. His autohemolysis was markedly increased and not corrected by glucose. These findings resemble the type 2 of non spherocytic hemolytic anemia [34]. It is tempting to suggest that the severe clinical condition resulted from an interaction of the two enzymatic defects coexisting in his red cells. A possibility of a potentiating detrimental effect of a double enzymatic abnormality has been suggested by Oski *et al.* [35] who described a mild hemolytic anemia and abnormal autohemolysis in a woman heterozygous for G6PD and pyruvate kinase deficiency.

Summary

A new genetic defect of red cells, namely deficiency of adenylate kinase, has been detected in an Arab family with coexistent deficiency of glucose-6-phosphate dehydrogenase due to an electrophoretically fast mutant. The adenylate kinase deficiency was transmitted most

probably by an autosomal gene with partial and variable expressivity in heterozygotes. The propositus with deficiency of both enzymes presented a congenital non-spherocytic hemolytic anemia with marked reticulocytosis and increased autohemolysis not correctable by glucose. H older sister with adenylate kinase deficiency only showed moderate anemia with very slight reticulocytosis, mild hyperbilirubinemia and slightly increased autohemolysis corrected by glucose. A causative relationship between the adenylate kinase deficiency and the anemia has not been yet established with certainty

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Authors' address: Prof. Dr. Arieh Szeinberg, Sara Gavendo, M. Sc., Jacob Zaidman, M. Sc., Judith Ben-Ezzer, M. Sc., Department of Chemical Pathology, Government Hospital, *Tel-Hashomer* (Israel); Dr. David Kahana, Department of Pediatrics, Government Hospital, *Hadera* (Israel)

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V. Chini, L. Bonomo and C. Sirtori: **Proceedings of the International Symposium on Gammapathies, Infections, Cancer and Immunity.** Carlo Erba, Milano 1968. 94 p.

Auf Einladung der Carlo Erba-Foundation fand am 30. September 1967 in Bari ein Symposium statt, an welchem 18 Autoren die vielschichtigen Beziehungen der Immunreaktionen zu Infektionen, Autoimmunität und maligner Entartung diskutierten. Die im *vorliegenden Band zusammengefassten 12 Vorträge geben denn auch einen Einblick in* die Komplexität der Grundfunktionen des immunologisch aktiven Zellsystems. Der schmale Band vermittelt auch dem erfahrenen Immunbiologen so zahlreiche Anregungen, dass seine Anschaffung wärmstens empfohlen sei. A. Hässig, *Bern*

Actes du VI^e Congrès National de Transfusion Sanguine. Tours, 22–26 juin 1966, tome I et II; supplément au tome II, No 3 de la Revue de Médecine de Tours, mai/juin 1968. La Simarre, Tours (France). 873 p.

Der unter E. Chatillon herausgegebene Bericht des VI. französischen Transfusionskongresses, organisiert von der französischen Gesellschaft für Bluttransfusionen unter Mitwirkung der nationalen Gesellschaften für Hämatologie und Pädiatrie sowie des französischen Roten Kreuzes umfasst im ersten Teil Referate über folgende Gebiete: 1. Zelluläre und humorale Immunität inkl. Transplantationsimmunität mit Übersichten sowie experimentellen Arbeiten über die γG-Immunglobulinstruktur und die Immunglobuline des Kolostrums; 2. Präparation von Immunglobulinen, speziell von Hyperimmunglobulinen und lyophilisierbaren Präparaten und deren klinische Verwendung sowie Methoden zur Titerbestimmung antibakterieller und anti-viraler Antikörper in diesen Produkten. 3. Immunglobulinopathien mit ausgewählten kasuistischen Fällen von speziellen Dys- und Paraproteinämien sowie hereditäre und erworbene Immunglobulinmangelzustände, ferner Vorschläge für quantitative Immunglobulinbestimmungen (passive Hämagglutination); 4. Therapeutische Verwendung von Immunglobulinpräparaten bei bakteriellen Infekten und Prophylaxe von Viruskrankheiten sowie die Möglichkeit der Unterdrückung allergischer (anaphylaktischer) Reaktionen durch unspezifische Immunglobuline der Reaginklasse; 5. Organisatorische Probleme im Transfusionswesen mit Beschreibungen von Organisations- und Datenverarbeitungssystemen in einer Reihe von französischen Blutspendezentren. 6. Probleme der Blutgruppenserologie, namentlich experimentelle Arbeiten über seltene Phänotypen im ABO-System, seltene Erythrozytenantikörper, Leukozyten- und Thrombozytenantikörper sowie eine immunologisch interessante Beobachtung über ABO-Blutgruppensubstanzen und Immunglobulinantigene im allergenischen Extrakt von Hausstaub. Im zweiten Teil wird über die folgenden 8 Kolloquien berichtet: 1. Serumgruppen und Enzympolymorphismen. 2. Konservierung von Blut und Blutprodukten, speziell von Problemen des Überlebens von Zellelementen. 3. Hämodynamik und Metabolismus von Blutkomponenten, insbesondere Fragen der Messung dieser physiologischen Prozesse. 4. Automation im Blutspendezentrum (Automaten für Blutgruppenbestimmungen, Lues-Screening, Transaminase-Screening). 5. Hämolytische Erkrankung des Neugeborenen, namentlich Fragen der Diagnose dieser Krankheit in der Amnionflüssigkeit und Probleme der intrauterinen Transfusion. 6. Hämorrhagischer Schock, seine Pathogenese und Therapie. 7. Überlebensstudien an Thrombozyten mit einer Reihe von experimentellen Beiträgen zur Ausarbeitung standardisierbarer Kriterien und 8. Probleme der Spenderwerbung. Leider wird die Orientierung in den beiden Bänden durch das Fehlen eines Inhaltsverzeichnisses erschwert. Einige ausgezeichnete Übersichtsreferate sowie eine Reihe von experimentellen Originalarbeiten machen jedoch das Studium dieses Kongressberichtes empfehlenswert. Bütler, *Bern*

We have had the opportunity to evaluate blood proliferocytes during human malaria. Increased numbers of proliferocytes were found in blood from 5 malaria patients. This result led us to carry out experimental studies on blood proliferocytes in experimental animals.

Materials and Methods

Blood labeling, micromethod. Peripheral blood was obtained from patients' fingertips. Animal studies utilized blood from the excised tail tip or from cardiac puncture. White cell counts and stained blood smears were routinely obtained. All blood specimens (each about 0.2 ml total volume) were drawn into heparinized hematocrit microtubes and mixed with 0.01 ml ^{3}H-thymidine in saline (100 μc/ml, 15 c/mM, New England Nuclear). The blood tubes were then incubated for 1 h at 37 °C to permit DNA labeling. After brief centrifugation to sediment the leukocytes, the segment of each microtube containing the column of buffy coat with part of the upper layer of red cells was cut away. The contents of each leukocyte rich segment was delivered into a siliconized spot plate and mixed with a drop *of normal saline to resuspend the cells. This buffy coat mixture was then loaded into a fresh* microtube. The loaded end of the tube was rapidly drawn across a clean glass slide so that a narrow linear smear of the leukocyte suspension was obtained. The slide smears were processed for autoradiography (ARG), exposed for one week, then developed and stained as previously described [7] except that Kodak NTB-2 emulsion diluted 1:1 with water was used here. At least 1,000 mononuclear cells were examined in each slide and the number of proliferocytes found was recorded. All proliferocytes had 10 or more grains overlying their nuclei. ARG background was always much less than 3 grains per equivalent cell area. Many *proliferocytes were heavily labeled so that fine detail was obscured. Partial classification* of less labeled blood proliferocytes was attempted. The percent proliferocytes found per total mononuclear cells examined was calculated. The results were expressed as the proliferocyte index, i.e., the calculated number of proliferocytes per 10,000 mononuclears.

Adult male mice, HA/ICR strain, weighing about 20–25 g were infected with *P. berghei* by i.p. injection of 0.5 ml malarial mouse blood on day 6 which had been diluted 1/200 with normal saline. This dose routinely transmits malaria and kills 95 to 98% of 1-month old mice by the sixth day[1]. Mice were sacrificed each day after inoculation and blood obtained for proliferocyte studies. Another group of mice received 0.5 ml of a 1:10 saline dilution of malaria blood which had first been refrigerated for 3 days to kill all organisms.

Adult Fisher strain rats were used in other experiments. Splenectomy was carried out in both rats and mice following ether anesthesia. Sham splenectomy was performed in some animals by merely removing parts of omentum.

Results

Blood proliferocyte levels of 10 healthy human adults (ages 21 to 45) ranged from less than 10 to 20 proliferocytes per 10 000 mononuclear cells with a mean value of 5. The labeled cells seen in their

[1] Unpublished data.

blood consisted mostly of large lymphocytes and monocytes with clear or pale cytoplasm Other rarer proliferocytes resembled young lymphocytoid monocytoid, and plasmacytoid cells with deeply basophilic cytoplasm. No small lymphocytes were found labeled

Five recent veterans of Vietnam (ages 21–25) were seen when they were febrile and in the midst of an attack of Vivax malaria. All had positive blood smears for malaria and were promptly treated after diagnosis. None remained febrile more than 3 days Distinctly increased numbers of proliferocytes as much as 20-fold greater than normal were seen in blood from these malaria patients at the onset. Only one of the patients had a palpable spleen. None manifested prominent hemolysis or anemia. After clinical response to therapy within 3 to 4 days, blood proliferocyte levels rapidly declined The types of blood proliferocytes seen during their illness included all cell types seen in normal blood No small lymphocytes were seen labeled

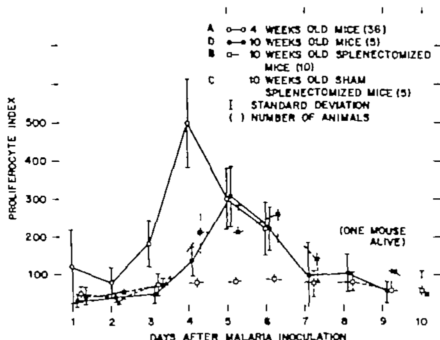


Fig 1 Blood proliferocytes during mouse malaria. Group A 4 weeks old mice; group B 10 weeks old splenectomized mice; group C 10 weeks old sham splenectomized mice group D 10 weeks old non-operated mice.

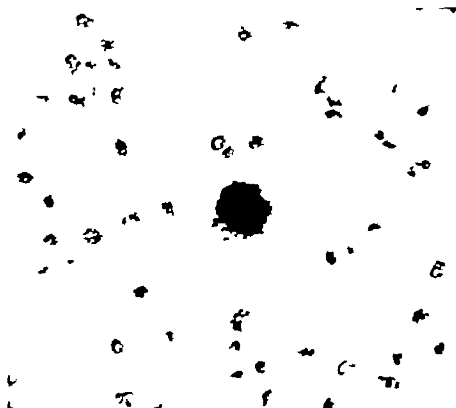


Fig. 2. Autoradiograph of mouse peripheral blood on day 6 after malaria inoculation. Note the labeled small lymphocyte (proliferocyte) in center and high degree of red cell parasitism.

Studies of blood proliferocytes in normal and malarial mice were carried out. Blood proliferocyte levels in normal mice were higher than in normal humans: in a group of 53 normal mice the mean blood proliferocyte index was 38 with a standard deviation of 22. Labeled cells seen in normal mouse blood appeared to be both small and large lymphocytes, as well as monocytes.

Thirty-six young mice were inoculated with malarial mouse blood. Blood proliferocyte levels were monitored daily thereafter. The result of this experiment is shown in figure 1 (group A). Two hours after inoculation with malarial blood, proliferocyte levels remained within control limits. Subsequently, proliferocytes increased during days 1–3. Maximal blood proliferocyte levels were seen on day 4 in all mice

when more than a 10-fold rise was noted (mean value 490) This high blood proliferocyte was not entirely sustained for on days 5 and 6 blood proliferocyte levels were 300 and 225 respectively All animals were severely anemic on days 5 and 6 with hematocrits ranging from 12 to 32 % and were barely alive when sacrified Red cell parasitism in the infected mice on day 4 was 9 . By day 5 50 of the red cells and at the day of death, 98 were parasitized with organisms. An autoradiograph prepared from mouse blood on the sixth day after inoculation with malaria is shown in figure 2 A high degree of red cell parasitism is seen. A mouse blood proliferocyte is shown which by its size, nuclear shape and scent pale cytoplasm, most closely resembles a lymphocyte.

The mean blood leukocyte count during experimental malaria in these mice initially was 15 000 mm^3 but declined to the lowest value on day 3 when the mean WBC count was 5 500 mm Afterwards, the WBC count *gradually* recovered Splenic hypertrophy consistently occurred during the malarial infection and was distinct on day 3 At day 6, all malarial mouse spleens had enlarged to approximately twice normal size

In another experiment, mouse malarial blood containing large numbers of killed parasites was injected into 4 mice Proliferocyte levels in tail vein blood remained within normal limits throughout 10 days after inoculation and all mice remained healthy Splenomegaly did not occur

The role of the spleen on blood proliferocyte levels during murine malaria was also evaluated in a group of somewhat older mice (10 weeks old) These results are depicted in figure 1 These older animals had either undergone splenectomy (group B) or sham splenectomy (group C) 6 weeks prior to malaria inoculation A third group was intact (group D) All animals were injected with murine malaria blood as before Older mice with intact spleens given malaria (group D) were slightly more resistant to the usual inoculum these animals survived for 9–10 days. Blood proliferocytes were also increased with a peak on day 5 during their infection. When 10 presplenectomized mice were given malaria (group B) the course of the infection was distinctly milder and the mice lived for 12 days. Blood proliferocytes barely increased in this group and no peak levels were seen. A third group of 5 mice (group C) who had been previously sham splenectomized, responded to malaria in a fashion similar to the older intact group

The previous results have shown that malarial infection is associated with increases in blood proliferocytes The effect of splenectomy alone on blood proliferocyte levels in mice and rats without interaction of malaria was studied. These results are shown in figure 3 Twenty mice were splenectomized and the proliferocytes in the blood serially evaluated Splenectomy resulted in a rise in blood proliferocytes to a peak at 14 days post operation The blood proliferocyte levels remained elevated for 2 weeks then gradually fell to normal levels by 6 weeks. Sham splenectomy in 10 mice did not perturb their blood proliferocyte levels.

A group of 10 rats also underwent splenectomy and blood proliferocytes were monitored post operation. These results are also shown in figure 3 These animals, like the mice showed a gradual rise in blood proliferocytes with distinct elevated levels on day 7 which rose further at day 14 to a mean of 125 then gradually declined In contrast, blood proliferocyte levels in normal intact rats had a mean value of 55

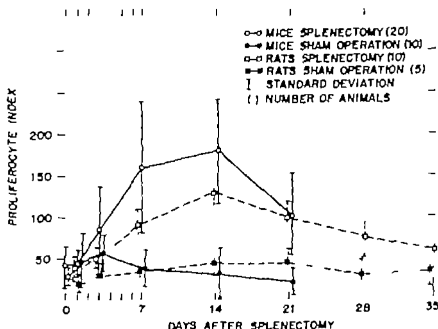


Fig 3. Blood proliferocytes following splenectomy and sham splenectomy in mice and rats (no malaria)

Since anemia is an important aspect of murine malaria the possibility that the blood proliferocyte response was mediated by the anemia, *per se* was explored. Two adult rabbits were acutely bled via cardiac puncture so that their hematocrits fell to 50 pre-treatment values. Daily blood proliferocyte levels did not show any significant change occurred in spite of subsequent reticulocyte responses as high as 20 A group of rats were made severely anemic after receiving phenylhydrazine In spite of normal reticulocyte responses, blood proliferocyte levels failed to increase.

Discussion

The present report is a quantitative study of DNA synthesizing cells of the blood. These cells are here named proliferocytes. A proliferocyte is any cell having the capcaity of promptly utilize ^{3}H-thymidine for its ongoing DNA synthesis so that it labels promptly and is seen in prepared ARG Excluded from this definition are unlabeled but otherwise identical appearing cells of any tissue of fluid which are in other phases of their cell cycle at the time of labeling and which may or may not also proliferate eventually The present definition permits the detection and quantitation of proliferocytes in tissues or fluids at the time of testing While numerous studies of cell proliferation and kinetics have utilized H-thymidine to study proliferocytes and their progeny in generative compartments, few workers have evaluated proliferocytes in non-generative and transit compartments such as blood, urine and various secretions The presence of proliferocytes in these areas in health and disease deserves further study

The present study shows that blood proliferocytes are increased when human beings and mice are infected with malaria. The types of blood cells seen labeled here do not seem to differ from those seen in viral and bacterial infections previously reported to show increased blood proliferocytes. The rarity yet diversity of blood proliferocytes makes their morphological classification difficult JANDL has shown that splenic cell proliferation is stimulated by splenic work either due to increase cell trapping or induced immunologic challenge [9] Blood proliferocytes during malaria may thus represent spillage from the hyperplastic spleen during stimulation by work. Recently however CHAN *et al.* [10] reported that the number of H-thymidine labeled

cells (proliferocytes) detected in normal rat blood increased as much as 4.5 fold 7 days after splenectomy. Increased blood proliferocytes persisted in their splenectomized rats for as long as 23 days; our results in mice and rats confirm and extend their findings. Compensatory hypertrophy of remaining lymphoid organs after splenectomy was suggested as a cause for the results seen. Presumably proliferocytes are shed into the blood during this process. This hypothesis is quite compatible with splenic hypertrophy as a source of increased blood proliferocytes in malaria. Our data suggest overall that blood proliferocytes arise from stimulation of diverse lymphoid tissues. No specific splenic role is envisioned in blood proliferocyte production except that the spleen is a major source of lymphoreticular tissue. In splenectomized animals, compensatory lymphoreticular hyperplasia with increased blood proliferocytes could ensue.

Summary

Tritiated thymidine labeling of blood leukocytes *in vitro* was used to detect circulating mononuclear blood cells capable of proliferation. Blood proliferocytes were found to be markedly elevated acutely in 5 patients with malaria. Mice infected with murine malaria demonstrated maximally increased numbers of blood proliferocytes 4 to 5 days after inoculation. Prior splenectomy in mice prevented the usual proliferocyte increase of malaria. Splenectomy alone in mice and rats yielded increased numbers of blood proliferocytes in the absence of malaria. Blood proliferocytes seem to represent 'spillage' from lymphoreticular tissues stimulated by immunologic or reticuloendothelial work.

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Authors' address: Dr. Joseph R. Rubini, Dr. Kenoo Matsuo and Dr. Lee Rane, Department of Medicine, University of Miami School of Medicine, *Miami, Fla–33136* (USA)

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The Clinical Laboratories, Section of Cytochemistry Meir Hospital, Kfar-Saba, and Department of Microbiology Bar Ilan University Ramat-Gan

Enzymatic and Chemical Changes in Human Lymphocyte Cultures

L. A. Rozenszajn and Dina Fischer

The normal population of lymphocytes in human blood consists predominantly of small cells, a few large lymphocytes and occasional atypical mononuclear cells [1] Phytohemagglutinin (PHA) an extract of *Phaseolus vulgaris* causes agglutination of the red cells in peripheral blood cultures [2] and stimulates the transformation and mitosis of the lymphocytes [3] Neutrophils and monocytes show signs of degeneration while eosinophils retain their morphological characteristics. There is a difference of opinion as to the origin of macrophages. Some authors believe that macrophages originate from lymphocytes [4 5 6] while others, that they are derived from monocytes [7] Cytochemical investigations of cells in blood cultures during the process of transformation have been reported by various authors [6 8–14]

In the present study we tried to examine and estimate both qualitatively and quantitatively the activity of various enzymes and chemical constituents in human blood cell cultures (BCC) We were especially interested in the biochemical changes taking place during the transformation of lymphocytes stimulated by PHA.

Materials and Methods

Using the method of Moorhead *et al.* [15] with slight modification [16] BCC were made from peripheral blood of 14 healthy subjects. Using the same method pure lymphocyt cultures were made after the lymphocytes had been separated on glass columns [7].

Light and phase contrast microscopy were used. Fluorescent microscopic studies were done with acridine orange (AO) for RNA and DNA while neutral red (NR) was used for neutral fats [17]

Cytochemical examinations were performed on the cells after 24, 48, 72 h of culture. As control, blood cells cultured without PHA were used. The cytochemical staining was done immediately after the smears were prepared from the BCC.

The rate of transformation of lymphocytes was determined by counting 200 cells in smears which were stained with MGG staining. Small lymphocytes, intermediate cells and blast-like cells, were counted.

Staining methods used included May-Grünwald-Giemsa (MGG), peroxidase for general diagnosis [18], azo-dye coupling method for acid phosphatase (AP) [19] and for alkaline phosphatase activity [20]. Esterases were determined by using alpha-naphthyl acetate, naphthol AS-D chloroacetate and naphthol AS-D acetate as substrates [21]. Dehydrogenases: glucose-6-phosphate dehydrogenase (G-6-PDH), lactic dehydrogenase (LDH), succinic dehydrogenase (SDH) [22] and dihydroorotate dehydrogenase (DHO.DH) [23]. The latter was demonstrated using the same technique as for G-6-PDH, the incubating solution containing equal amounts of dihydroorotate 1 mg/ml and Nitro BT 0,5 mg/ml of Sörensen buffer м/15 pH 7.4. Diaphorases: dihydronicotinamide-adenine dinucleotide diaphorase (NADH diaphorase) and dihydronicotinamide-adenine dinucleotide phosphatate diaphorase (NADPH diaphorase) [22].

For the study of DNA metabolism ^{3}H-thymidine was used in a concentration of 1 μc/ml. Autoradiograms were prepared using Ilford K 5 emulsion. The exposure period was 7 days. Methyl-Green-Pyronin (MGP) staining for DNA and RNA was used with the adaptation of Kurnick's method [24]. Sudan Black B [25], Sudan III [17], Oil Red O [26] were used for investigation of lipids. Periodic acid Schiff (PAS) reaction was carried out with and without ptyaline digestion [27]. PAS staining was also done on smears which were prepared from heparinized venous blood of 9 healthy subjects. The blood was incubated in sterile glass tubes for 24, 48 and 72 h with the sole addition of glucose to make up 100, 500 and 1000 mg % solutions, no other constituents being added. A control venous blood was incubated without glucose.

The activity of the acid phosphatase, dehydrogenases, diaphorases and the glycogen content in the lymphocytes was estimated in a semiquantitative way [19, 22, 28].

The glycogen content and the acid phosphatase activity were determined by counting 100 small lymphocytes and 100 transformed lymphocytes (intermediate and blast-like cells). The glycogen content of lymphocytes incubated with glucose, the rate of DNA synthesis and the enzymatic activity of dehydrogenases and diaphorases were determined by evaluating the score in 100 lymphocytes.

The grading for DNA synthesis (^{3}H-thymidine) was done according to the number of grains present in the nucleus of the cell: 0 = nucleus containing no grains, 1+ = nucleus containing isolated groups of grains, 2+ = nucleus rich in grains but with certain areas void of them, 3+ = nucleus overcrowded with grains.

Results

Morphology

Phase contrast microscopy. After 24 h of culture, intermediate cells with reticular nuclei and a more abundant cytoplasm replaced the small lymphocytes. A few cells had visible nucleoli, and some exhibited movement. After 48 h the cells were large with nuclei which contained

a nucleolus. Mitochondria and round shiny bodies 1 μm in diameter were distinguished in the cytoplasm. After 72 h the majority of cells looked like blasts, some being large, others smaller while mitotic figures were also observed. Macrophages and remnants of neutrophils were already noted after 24 h The eosinophils retained their morphological characteristics. In the culture of pure isolated lymphocytes no macrophages were seen.

May-Grünwald-Giemsa staining The cytoplasm of the transformed cells stained pale to dark blue Vacuoles which correspond to the shiny round bodies observed under phase microscopy were found in many cells. Nucleoli could be distinguished in the nuclei of blasts.

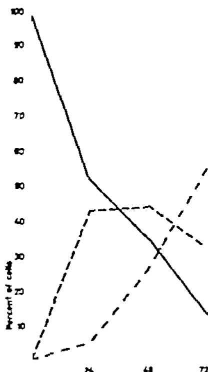


Fig 1

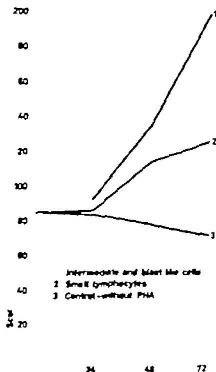


Fig 2

Fig 1 Differential count in leukocyte culture, stimulated by PHA. —— Small lymphocytes, intermediate lymphocytes, blast-like cells.

Fig 2 The score of the acid phosphatase activity in lymphocytes, in PHA-treated cultures, and in control cultures without PHA.

There was no difference between the rate of transformation of lymphocytes in the pure lymphocyte culture and in the blood cell culture (fig 1)

AO fluorescence The nuclei emitted a green fluorescence. In the cytoplasm of the majority of lymphocytes small bodies of red fluorescence could be distinguished The number of these fluorescent bodies was greater in transformed cells. In the macrophages the cytoplasm emitted a strong red fluorescence

NR fluorescence The spherical cytoplasmic inclusion bodies emitted a light green fluorescence while the nuclei and the cytoplasm showed an orange fluorescence The identity of the spherical refractile inclusion and fluorescent bodies could be demonstrated by staining with NR and examining the same cell both by phase contrast and fluorescent microscopy

Cytochemical Studies

Peroxidase staining Only neutrophils and macrophages showed enzymatic activity

Acid phosphatase Azo-dye staining was positive in lymphocytes in all stages of their transformation The enzymatic activity increased progressively and reached a peak after 72 h The small lymphocytes that did not undergo transformation in the culture with PHA showed a stronger enzymatic activity than the lymphocytes cultured without PHA (fig 2) The vacuoles stained brown in a different way from the specific stain of the enzyme (fig 3) The staining took place also when the media did not contain the specific substrate The enzymatic activity was very strong in macrophages.

Alkaline phosphatase No enzymatic activity was observed in the lymphocytes.

Esterase activity Using alpha naphthyl acetate as substrate the enzymatic activity in the small lymphocytes was in the form of small brown bodies in the cytoplasm or small scattered granules. In the blasts many small cytoplasmic granules were seen while in the monocytes and macrophages the enzymatic activity was strong taking the form of aggregates and numerous granules. Using naphthol AS-D chloroacetate as substrate the neutrophils exhibited strong enzymatic activity in the form of purplish-red clumps scattered in the cytoplasm. Lymphocytes were not stained and in the macrophages a few large





Fig 3 Fig 4

Fig 3. Acid phosphatase activity in PHA-induced lymphocytic cells. The activity is demonstrated by the scattered granules in the cytoplasm. Some vacuoles are stained as round bodies with the azo-dye.

Fig 4 Transformed lymphocytes after 72 h in culture, showing strong activity of NADH diaphorase.

clumps could be distinguished. Using naphthol AS-D acetate as substrate the activity was observed in the lymphocytes in all stages of their transformation. In macrophages and neutrophils it took the form of reddish cytoplasmic granules.

Dehydrogenases (DHO DH SHD LDH G-6-P DH) *and diaphorases* (NADPH NADH) The enzymatic activity was observed in the form of diffuse cytoplasmic staining and/or bluish granules. Blasts and intermediate cells showed stronger activity than the small lymphocytes (fig 4 5)

Autoradiography The incorporation of thymidine ^{3}H corresponds to the synthesis of DNA and increases with the transformation of the lymphocytes (fig 6)

Methyl green pyronin The presence of RNA was made evident by a red staining which was stronger in the intermediate lymphocytes and strongest in the blasts.

With *Sudan III* and *Oil Red O* the cytoplasmic vacuoles stained red

By staining with *Sudan Black B* only a few black granules were seen in the cytoplasm in some small or transformed lymphocytes. Neutrophils and macrophages stained black.

Periodic acid Schiff In lymphocytes the staining increased in intensity in the first 24 h, and subsequently there was a reduction in staining intensity (fig 7) In some cultures there was a further increase in glycogen content in the second 24 h. In venous blood samples incubated with glucose only there was a progressive increase in the glycogen content during the 72 h of culture The glycogen content of cells depends on the amount of glucose present in the media (fig 7) In the smears that were treated with ptyaline PAS staining was negative

In the pure lymphocyte cultures, cells showed the same cytochemical characteristics as the lymphocytes cultured from whole blood

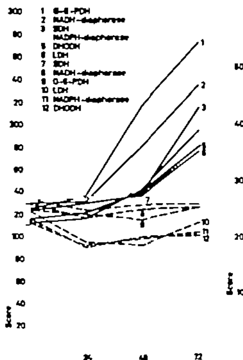


Fig 5

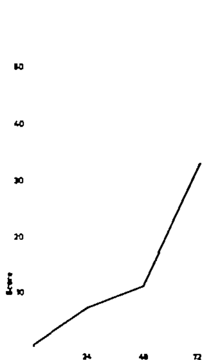


Fig 6

Fig 5. The score of dehydrogenases and diaphorases in the lymphocytes in PHA-treated cultures (——) and in control cultures without PHA ( ).

Fig 6. The score of DNA synthesis demonstrated by ³H-thymidine incorporation (autoradiography) in lymphocytes from leukocyte culture treated with PHA.

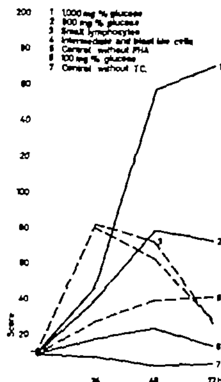


Fig 7 The score of the glycogen content of lymphocytes in PHA-treated BCC, of lymphocytes from blood incubated without PHA, and from blood incubated without culture medium and PHA, but with the ddition of different amounts of glucose.

Discussion

The present study of the cytochemical changes accompanying morphological development, reveals in part the biochemical processes occurring in the transformation of lymphocytes

One of these processes, the metabolism of sugars expresses itself firstly in the accumulation of glycogen in the culture during the first 24–48 h followed by its decay during an additional day (fig 7) and secondly in an increase in the activities of the dehydrogenases and diaphorases involved in glycolysis (fig 5) The utilization of glycogen as an energy pool is higher in the cultures with blast transformation than in the lymphocytes from blood incubated without culture medium, because the increased metabolic activity of the first requires more energy

Examination of the glycogen synthesis in lymphocytes from blood incubated in glucose solutions without PHA indicated that the high level of glycogen synthesis is independent of the lymphocyte transformation and the degree of synthesis depends on the amount of sugar provided to the incubating blood. There are differences in the degree of synthesis of glycogen in lymphocytes from different donors, both when the blood is incubated with sugar only and when it is incubated in cultures in the presence of PHA. The rate of transformation is perhaps better when the synthesis of glycogen is higher

An increase in the activity of DHO.DH which participates in pyrimidines synthesis indicates that synthesis of nucleic acids takes place (fig 5) This was corroborated by the demonstration of increased DNA synthesis (fig 6) and RNA content (MGP) in the cytoplasm of lymphocytic cells.

With the cytochemical staining method used we could not detect any signs of transformation of lymphocytes into cells of other series.

Similar to the findings reported in the normal hemopoietic cells, AP activity was stronger in the intermediate and blast like cells than in the small lymphocytes [19] The increase in AP activity in the small lymphocytes as compared with its activity in the cells cultured without PHA (fig 2) is probably due to the effect of PHA on the permeability of the lysosomal membrane [29]

The vacuoles seen in the transforming cells are probably the products of lysosomal autodigestion We believe that the vacuoles are an early sign of cellular damage in the same way that the presence of lipid inclusions occurring in leukocytes in synovial fluid is evidence of white cell degenerative changes [30] These vacuoles which have been described as containing neutral fats [10] stained selectively with Sudan III, Oil Red O and exhibit fluorescence with NR, thus showing the cytochemical characteristics of lipids presumably neutral fats. The faint staining of the vacuoles by the azo dye technique for AP both with and without substrate, indicates a false reaction resulting from the solubility of Fast Garnet dye in lipids.

The macrophages which are not present in the pure lymphocyte cultures, appear in BCC as of monocytogenous origin. Similarly to the blood monocytes they show the same strong activity of AP and esterase alpha naphthyl acetate, a positive staining of peroxidase and Sudan Black B. The strong AO fluorescence is probably due to its own lysosomes and that of the phagocytozed cytoplasm of the neutro-

phils. The phagocytic activity of macrophages was demonstrated also by the finding within them of the remnants of cytoplasm of the neutrophils, made evident by the positive reaction of naphthol AS-D chloroacetate esterase. This enzyme is found only in the cytoplasm of granulocytes.

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Summary

In lymphocytes from healthy subjects, cultured *in vitro* in the presence of phytohemagglutinin, qualitative and semiquantitative evaluations of enzymatic activity and chemical constituents were made. The metabolism of sugars expresses itself by the accumulation of glycogen and by an increase of the dehydrogenases and diaphorases involved in glycolysis. DNA and RNA synthesis was made evident. The activity of acid phosphatase increased with the transformation of the lymphocytes, being stronger in the transformed lymphocytes than in the small ones. The vacuoles which appeared as shining bodies when examined under phase contrast microscopy appeared to contain neutral fats.

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Authors' address: L. A. Rozenszajn, Ph. D., Head of Clinical Laboratories; Dora Fischer, M. Sc., Laboratory of Hematology, Meir Hospital, *Kfar-Saba* (Israel).

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Departments of Medicine (Head: Prof. P. [illegible]), Medical Microbiology (Head Prof. E. Mustakallio) and Pediatrics (Head Prof. T. Peltonen) University of Turku, and the Communal Geriatric Hospital of Tampere (Head R. Heikinheimo)

Transketolase Activity of Red Blood Cells from Infancy to Old Age

T. Markkanen, R. Heikinheimo and M. Dahl

On the basis of current knowledge, the transketolase activity (TKA) of the red cells falls in thiamine deficiency [3–13 15 16, 22–24 27] and increases in megaloblastic anemia caused by vitamin B_{12} deficiency [19 20] During experimental hemolysis the TKA of the red cells tends to increase, apparently due to the presence of numerous young forms of red cells [1 14] The TKA of foetal red cells has been found to exceed that of the maternal cells [26]

The present study was carried out to investigate the TKA changes in man in different age groups from birth to late old age

Material and Methods

The series comprised 414 subjects. Detailed information concerning them is given in table I, which carries the same group numbers as figure 1

The series was composed and initially examined in hospital conditions. The children of the series were found in the Department of Obstetrics and/or Pediatrics where they were under observation. They were all in an excellent physical condition and had no diseases which current opinion might hold to affect the transketolase activity of the red cells. The adult series was compiled from the University Department of Medicine and from the Geriatric Hospital. The young and middle-aged patients were under observation for some minor internal complaints and had no deficiency diseases, or disorders which according to current opinion might affect the TKA of red cells. The geriatric series contained the following diagnoses arthrosis, chronic rheumatoid arthritis and mainly cardiovascular diseases. All these patients were in an excellent nutritional condition.

All specimens were taken from venous blood and collected during the same season of the year (autumn) from the whole series. The specimens were analyzed as large series immediately after they had been drawn, by method described previously [15]. The TKA unit chosen for the present study was µmol sedoheptulose-7-phosphate/ml cellular mass/30 min, 37°C, pH 7 4 (international unit).

Table I. Presentation of the total series studied

Group	Age	Men	Women	Total	Weight, kg mean ± SD	Hb, g/100 ml mean ± SD
1	0– 7 days	19	12	21	3.450 ± 1.05	16.4 ± 2.8
2	8– 30 days	4	6	10	3.580 ± 0.28	16.3 ± 2.4
3	31 364 days	5	3	8	5 400 ± 1 48	12.5 ± 1.0
4	1– 9 years	5	9	13	20.1 ± 8.1	12.2 ± 1.5
5	10– 19 years	8	11	19	50.6 ± 19.2	13.6 ± 1 1
6	20– 29 years	13	8	21	66.9 ± 12.6	13.7 ± 2.2
7	30– 39 years	12	6	18	73.0 ± 13.6	14.2 ± 1.5
8	40– 49 years	15	11	26	74.5 ± 14.0	14.6 ± 1.8
9	50– 59 years	8	13	21	73.0 ± 12.3	13.7 ± 0.9
10	60– 69 years	10	22	32	65.0 ± 13.7	12.9 ± 2.7
11	70– 79 years	20	79	99	58.0 ± 13.3	13.4 ± 1.8
12	80– 89 years	10	85	95	52.0 ± 9.0	13.6 ± 1 1
13	90– 99 years	2	19	21	48.0 ± 10.4	13.7 ± 1.5

Table II. TKA (units) in red blood cells in the whole series, men and women separately

Group	Whole material mean ± SD	Men mean ± SD	Women mean ± SD
1	5.31 ± 0.98	5.26 ± 1.08	5.39 ± 0.83
2	5.31 ± 1 17	5.70 ± 1 44	5.06 ± 1.00
3	5.14 ± 0.80	5.22 ± 0.97	5.00 ± 0.53
4	4.92 ± 0.53	5.23 ± 0.49	4.78 ± 0.52
5	4.88 ± 0.68	4.86 ± 0.67	4.90 ± 0.72
6	4.60 ± 1 15	4.31 ± 0.80	5.07 ± 1.50
7	4.88 ± 1.09	4.87 ± 1.02	4.90 ± 1.28
8	4.84 ± 0.85	5.07 ± 0.77	4.54 ± 0.90
9	5.09 ± 0.93	5.07 ± 0.60	5.09 ± 1 11
10	4.72 ± 0.98	5.06 ± 0.98	4.57 ± 0.96
11	4.61 ± 0.87	4.73 ± 0 86	4.57 ± 0.87
12	4.57 ± 0.93	4.64 ± 0.87	4.57 ± 0.94
13	4.43 ± 1 11	5.45 ± 0.49	4.52 ± 1.11

The statistical differences between the two sexes are not significant.

The results were treated by computer (Institute for Applied Mathematics, University of Turku). The overall mean and standard deviation (SD) of TKA were calculated by age groups (groups 1 13). The same values were calculated separately for both sexes by age groups (table II). In addition, regression analysis of all the data was carried out (fig. 1), the caption giving all the relevant details. The confidence limits taken for the regression analysis were ± 5.0%.

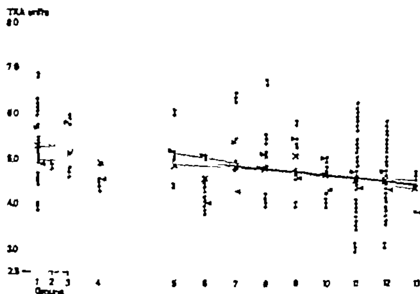


Fig. 1. Regression analysis of the TKA data of the total series. The group numbers are the same as in table I. The solid line is the line of regression, the thin lines indicate the 95 percent confidence region. The individual results for the test subjects are indicated by points, the group means by crosses, while the triangles indicate the upper and lower limits. Notice that the scale of the axis changes at the age of 10 years (group 5). The correlation coefficient is 0.2197, the F-value 0.7634. The hypothesis that the regression line runs horizontally leads to the T-value of –4.572.

Results

The results are presented in table II and figure 1. Table II shows that the red cell TKA was at its maximum in the newborn and exceeded, on the average, the 50 unit limit up to the age of one year. Subsequently the TKA gradually began to fall and was at its lowest in the last group aged 90–99 years. No statistically significant difference could be shown between the sexes. The regression analysis of all the data revealed that the regression curve was slightly declining throughout (fig. 1). The T value obtained was –4.572. Accordingly, the regression declines significantly towards the older age groups. Details of the statistical mathematics are presented in the table captions.

Discussion

The statistical and mathematical treatment of the results showed that the red cell TKA gradually decreases from birth towards late old

age. The variation of individual values within the age groups is large and numerous reading on either side of the mean are noted. It may be concluded from figure 1 that the differences really are on the statistico-mathematical level and although as such they are clearly significant (T = −4.573) from practical clinical work point of view the TKA differences between young and old age groups are relatively small.

The general view is that low TKA of the red cells indicates the absence of the co-enzyme in the patient. For this reason TKA determinations have been used to prove thiamine deficiency. All the members of the present series were in a good nutritional condition and had no symptoms which might have suggested a deficiency state. It may be asserted that in the geriatric groups (groups 11–13) spontaneous intake of food may be reduced, as is also indicated by the mean weight loss. All the members of these groups were, however, treated in a geriatric hospital, where their adequate nutrition could be actively controlled.

When the red cell ages the activity of its enzyme systems may fall [1, 17, 18, 21, 25]. The present study in fact disregarded the ageing of the red cell itself. The point is rather the ageing of the red cell's parent cell, and its effect on the quality of the red cells circulating in the blood. The question of interest was, therefore, the general effect of ageing on the biochemical life of the cell. The TKA of red cells seems to fall with advancing age. This is in agreement with the findings concerning some other enzymes of the hexose monophosphate shunt.

Summary

The transketolase activity (TKA) of the red cells was studied in 414 test subjects from birth to advanced old age. The mean red cell TKA was highest in the newborn group (5.31 ± 0.98 units) and lowest in the oldest age group (4.43 ± 1.11 units). The other mean values fall between these extreme values. A statistico-mathematical regression analysis resulted in TKA curve declining significantly from the young age groups towards old age (T = −4.572). No significant difference in the TKA of red cells was noted between the sexes in any of the age groups.

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Authors' addresses: Docent Dr. T. MARKKANEN, Department of Medical Microbiology; Dr. M. DAHL, Department of Pediatrics, University of Turku, *Turku*; Dr. R. HEIKINHEIMO, Communal Geriatric Hospital, *Tampere* (Finland)

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Institut de Biochimie, Université de Lausanne, Lausanne

Etude de la fixation non spécifique des immunoglobulines G sur les érythrocytes

I. Influence de la variation de différents paramètres

D. Des Gouttes et H. Isliker

Les immunoglobulines G (IgG) [7] se fixent non spécifiquement à la surface de cellules telles que les érythrocytes [1, 2, 4, 11, 12, 14, 16, 23] et de particules inertes telles que le latex [6].

Plusieurs hypothèses ont été émises concernant la fonction physiologique que possèdent les immunoglobulines adsorbées *in vivo* à la surface cellulaire [3]. Les travaux d'Ovary [22] et Halpern [13] ont permis de conférer à cette fixation non spécifique un rôle dans les processus de désensibilisation. Chez l'homme, Jeannet et coll. [16] ont observé une négativation du test de Coombs après l'injection i.v. de γG-globulines standard ou traitées à pH 4 chez un patient hypogammaglobulinémique souffrant d'une anémie hémolytique. Des expériences faites chez le chien et chez l'homme montrent la présence, à la surface des érythrocytes, d'une fraction d'IgG augmentant la viabilité de la cellule [10, 27]. Les mêmes auteurs observent qu'une autre fraction d'IgG fixée sur les leucocytes humains, augmente l'activité phagocytaire de ces cellules vis-à-vis du staphylocoque doré [10]. Enfin, l'étude de la fixation des macrophages et des monocytes sur les érythrocytes recouverts d'IgG permet de constater la transformation des érythrocytes en sphérocytes, ce qui favorise ainsi l'élimination de la cellule antigénique [18].

La nature de la fixation non spécifique des immunoglobulines à la surface cellulaire n'est pas encore connue, mais selon Cruchaud et coll. [8] la présence du fragment Fc serait nécessaire à la fixation cellulaire des γG-globulines. Pourtant, des expériences montrent que les

fragments Fab et Fc de l'IgG marquée à l'^{131}I se fixent non spécifiquement à la cellule en quantité égale, sur une base pondérale, à l'IgG native [12].

Le présent travail étudie *in vitro* les caractéristiques de la fixation non spécifique de l'IgG sur les érythrocytes lors de la variation successive de différents paramètres que comporte le système: nombre d'érythrocytes, quantité d'IgG mis en présence, conditions et mode d'incubation. L'existence d'une importante fixation de l'IgG sur le verre a conduit à mesurer parallèlement et à comparer la fixation de l'IgG sur le verre et sur les érythrocytes.

Matériel et méthodes

Préparation des érythrocytes. Les érythrocytes de lapin sont prélevés à raison de 10 à 20 ml par saignée à l'oreille. Le sang est recueilli dans un égal volume de solution d'Alsever stérile dont le pH est ajusté à 7,4 avec une solution d'acide citrique saturé. Sauf indication spéciale, les érythrocytes sont utilisés dès 24 h et jusqu'à 8 jours après leur prélèvement. Les érythrocytes humains sont prélevés chez les donneurs O Rh+ et traités de la même façon que les érythrocytes de lapin. Avant chaque expérience, les érythrocytes sont lavés 3 fois dans un volume de tampon égal à 15 fois celui des érythrocytes. La composition de la solution tampon, appelée «tampon ordinaire», est la suivante: un volume de tampon phosphate 0,15 M, pH 7,4 de conductivité égale à 8,52 mmho, mesurée à l'aide d'un conductomètre Radiomètre type CdM 2d, et 9 volumes d'une solution NaCl 0,15 M. La force ionique finale calculée est de 0,175. Le comptage des cellules est fait à l'aide d'un Coulter Counter Modèle A (Coulter Electronics Ltd., St. Albans, Herts., Angleterre) et l'on vérifie au microscope l'homogénéité de la suspension, l'absence de cellules leucocytaires et l'aspect des érythrocytes. Le degré d'hémolyse observé en cours d'expérience n'influence pas de façon significative la mesure de radioactivité des culots d'érythrocytes. Aussi, on négligera d'apporter une correction en rapport avec le degré d'hémolyse.

Préparation des IgG. Un mélange de sérums provenant de plusieurs lapins est précipité et lavé avec du sulfate d'ammonium saturé (concentration finale 40%). Les γG-globulines ainsi obtenues sont ensuite passées sur une colonne de DEAE-cellulose éluée avec un tampon phosphate 0,01 M, pH 7,4. Une préparation d'IgG hautement purifiée provenant de la Croix Rouge Hollandaise a aussi été utilisée. L'ultracentrifugation analytique de l'IgG faite à l'aide d'une ultracentrifugeuse Spinco modèle E, Beckman donne les caractéristiques de l'IgG suivantes:

$s_{20,w}$ = 6,7; D_w 20°C = 4,2 · 10^{-7}; $\bar{V}$ = 0,745; PM = 155.400. Les préparations d'IgG utilisées contiennent moins de 3% d'agrégats.

L'iodation des IgG est faite selon la méthode de Boca [3] modifiée par McConahey [20]. Le rendement du marquage varie de 20 à 50%, il diminue avec l'âge de la solution d'iode et dépend de la concentration en protéine. Les solutions d'IgG utilisées pour le marquage à l'iode radioactif ont une concentration variant de 0,1 à 3%. L'iode radioactif (^{125}I ou ^{131}I) stérile et sans entraîneur provient du Radiochemical Centre, Amersham (Angleterre) et de l'Institut fédéral de recherches en matière de réacteur, Würenlingen (Suisse). Après dialyse de la solution d'IgG marquée contre le tampon pendant 72 h, la quantité d'iode libre restant non fixée à l'IgG représente 1 à 2% de la quantité fixée à l'IgG. La radio-

activité est mesurée à l'aide d'un compteur à cristal Nuclear Chicago (modèle G 120-1). La concentration de l'IgG après le marquage est déterminée par la microméthode de Kjeldahl selon la technique rapportée par Kabat et Mayer [17] qui permet d'établir l'activité spécifique (cpm/µg de protéine) de l'IgG utilisée dans les expériences.

Les données numériques sont exprimées en moyenne ± l'erreur moyenne de la moyenne ($\bar{x}$ ± SE). Les significations de différences entre les moyennes sont calculées en utilisant le test t selon les méthodes statistiques habituelles [25].

La fixation non spécifique de l'IgG aux érythrocytes est effectuée en incubant pendant 60 min à 37°C l'IgG marquée à l'iode radioactif avec les érythrocytes préalablement lavés 3 fois dans un large excès de tampon ordinaire. Après l'incubation, le mélange est centrifugé à 2000 rpm pendant 10 min et le culot d'érythrocytes est lavé 3 fois dans 2 ml de tampon ordinaire. Dans certains cas, le lavage est fait avec une solution isotonique de glucose tamponnée avec le tampon ordinaire afin de diminuer l'élution de l'IgG fixée non spécifiquement. La quantité d'IgG fixée aux érythrocytes est mesurée par la radioactivité du culot d'érythrocytes lavés. La fixation de l IgG sur le verre est évaluée en transvasant au cours du troisième lavage le culot d'érythrocytes dans un autre tube. La perte d'hématie au cours du transvasage est négligeable. La fixation non spécifique de l'IgG aux érythrocytes est exprimée en µg par culot ou en molécules d'IgG fixées par culot ou par érythrocyte. La fixation de l'IgG sur le verre est exprimée en µg d'IgG fixés sur la surface de verre en contact avec le volume constant d'incubation.

Résultats

Des expériences ayant pour but d évaluer la reproductibilité du système montrent qu il n y a pas de variation significative de la fixation non spécifique de l'IgG avec différentes populations d érythrocytes ou avec des érythrocytes dont le temps de conservation diffère. Au cours du temps de conservation, l'IgG de lapin, contrairement à l IgG humaine [11] ne se fixe pas significativement plus sur les cellules.

Influence de la concentration des érythrocytes La variation de la fixation de l IgG-^{131}I en fonction du nombre d érythrocytes a été étudiée en diminuant progressivement jusqu à un facteur de 10 fois le nombre d érythrocytes mis en présence d une même quantité d IgG La figure 1 montre une augmentation significative du nombre de molécules d IgG fixées par érythrocyte à mesure que le nombre d érythrocytes diminue. Une semblable expérience a été faite en diminuant d un facteur de 100 le nombre d érythrocytes. Les résultats présentés dans le tableau I montrent que le nombre de molécules d IgG par érythrocyte augmente de façon exponentielle à mesure que la quantité d érythrocytes incubés diminue.

Influence de la concentration de l'IgG Des quantités croissantes d IgG-^{131}I ont été incubées avec un nombre constant d érythrocytes. L ex

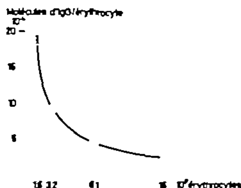


Fig 1 Variation de la quantité d'IgG-^{131}I fixé par érythrocyte en fonction du nombre d'érythrocytes incubés. Une quantité constante de 318 µg d'IgG-^{131}I est incubée avec un nombre croissant d'érythrocytes. Les µg d'IgG fixés par érythrocyte sont convertis en molécules en estimant le PM de l'IgG à 1,5 10^5 Le lavage des érythrocytes est fait avec une solution de glucose isotonique tamponnée avec le tampon ordinaire. Chaque point représente la moyenne de 5 valeurs ± l'erreur moyenne de la moyenne ($\bar{x}$ ± SE)

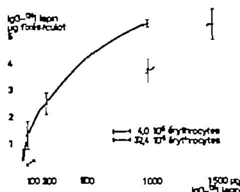


Fig 2 Variation de la fixation de l'IgG-^{131}I aux culots d'érythrocytes en fonction de quantités croissantes d'IgG-^{131}I. L expérience est faite avec deux concentrations d'érythrocytes différentes auxquelles on ajoute des quantités croissantes d'IgG-^{131}I. La quantité d'IgG-^{131}I fixée est exprimée en µg par culot d'érythrocytes. Chaque point représente la moyenne de 5 valeurs ± l'erreur moyenne de la moyenne ($\bar{x}$ ± SE).

périence a été faite avec deux concentrations d'érythrocytes différentes. La fixation cellulaire de l'IgG est mesurée en fonction de la concentration de l IgG-^{131}I *ajoutée* (fig 2) La *quantité* d IgG satu

Influence de la force ionique L adjonction, au tampon ordinaire, d une quantité croissante de glucose isotonique (6 7%) permet de faire varier la conductivité du tampon en gardant constante l osmolarité le pH et le volume d'incubation. La force ionique est exprimée en unités de conductivité (mM HO) Une même expérience a été faite avec du glucose hypertonique (20%) La figure 4 montre que la fixa tion de l IgG aux érythrocytes augmente de façon exponentielle à mesure que la conductivité du milieu d incubation diminue la fixa tion cellulaire de l'IgG est moindre avec l'adjonction d une solution hypertonique de glucose. La fixation de l'IgG sur le verre n est pas influencée par la force ionique.

Influence de la température. La fixation de l IgG-^{131}I aux érythrocytes est mesurée en fonction d'une variation de la température du milieu d incubation de 4 C à 37°C (fig 5) La fixation de l'IgG-^{131}I sur le verre est mesurée parallèlement. On voit que la fixation de l IgG ^{131}I aux érythrocytes est dépendante de la température et diminue très significativement ($p < 0\,001$) lorsque la température augmente. Par contre la fixation de l IgG au verre, en présence d érythrocytes, aug mente significativement ($p < 0\,01$) avec la température. En l absence d érythrocytes, la fixation de l IgG au verre reste constante lors de la variation de la température. Ces résultats montrent l existence d'une compétition entre les érythrocytes et le verre pour la fixation non spécifique de l IgG-^{131}I

Influence du temps d'incubation. Afin d'étudier la cinétique du processus de fixation de l IgG-^{131}I aux érythrocytes, on fait varier le temps d'incubation d abord de 5 min à 60 min (tableau II) puis de 30 min à 10 h (tableau III) On constate que la fixation de l IgG-^{131}I reste constante au cours des différents temps d'incubation observés la fixation non spécifique de l IgG aux érythrocytes est un processus qui s effectue dans les minutes qui suivent l adjonction de l IgG-^{131}I aux cellules. L équilibre de cette fixation est rapidement atteint et reste constant. Il en est de même pour la fixation de l'IgG-^{131}I sur le verre.

Influence du lavage des érythrocytes après l'incubation. Après l'incubation, les érythrocytes sont lavés avec différentes solutions de même pH mais de force ionique variable. Dans le tableau IV les résultats sont comparés avec ceux obtenus par le lavage avec le tampon ordinaire. Un contrôle de la fixation sur le verre est fait parallèlement. Les résultats montrent que l élution de l'IgG-^{131}I fixée aux érythrocytes

Tableau II. Fixation de l'IgG aux érythrocytes et au verre en fonction du temps d'incubation (de 5 min à 60 min)

Durée d'incubation min	µg d'IgG-^{125}I fixés par culot	µg d'IgG-^{125}I fixés sur le verre
5	1,89 ± 0,13	1,26 ± 0,12
10	2,08 ± 0,12	1,25 ± 0,10
20	1,72 ± 0,23	1,93 ± 0,22
30	1,57 ± 0,14	2,13 ± 0,20
60	2,36 ± 0,19	1,66 ± 0,11

200 µg d'IgG-^{125}I ont été incubés avec 32,4 10^6 érythrocytes. Valeurs $\bar{x}$ ± SE; nombre d'essais = 5.

Tableau III. Fixation de l'IgG aux érythrocytes et au verre en fonction du temps d'incubation (de 30 min à 10 h)

Temps d'incubation	µg d'IgG-^{125}I fixés par culot	µg d'IgG-^{125}I fixés sur le verre
30 min	1,00 ± 0,16	1,98 ± 0,13
60 min	1,19 ± 0,12	2,05 ± 0,07
5 h	0,81 ± 0,13	2,16 ± 0,17
10 h	1,07 ± 0,09	2,17 ± 0,16

150 µg d'IgG-^{125}I ont été incubés avec 32,4 10^6 érythrocytes. Valeurs $\bar{x}$ ± SE nombre d'essais n = 5.

varie suivant le tampon de lavage utilisé après l'incubation. Elle est d'autant plus importante que la force ionique du tampon de lavage est grande. La fixation de l'IgG-^{125}I sur le verre reste, par ailleurs, constante. La quantité d'IgG-^{125}I fixée aux érythrocytes dépend du nombre de lavages (avec le tampon ordinaire) faits après l'incubation (tableau V). Dans cette expérience, la fixation de l'IgG-^{125}I sur le verre est incluse dans les valeurs obtenues pour le culot. On constate une faible diminution à chaque lavage de la quantité d'IgG-^{125}I fixée aux érythrocytes; la quantité d'IgG-^{125}I éluée à chaque lavage diminue et tend à devenir constante.

Tableau IV Fixation de l'IgG-¹²⁵I aux érythrocytes et verre en fonction de différentes solutions de lavage

Solution de lavage	µg d'IgG-¹²⁵I fixés par culot	µg d'IgG-¹²⁵I fixés sur le verre
Tampon phosphate 0,15 M	$0{,}20 \pm 0{,}01$ $p < 0{,}01$	$0{,}98 \pm 0{,}03$
NaCl 0,15 M tamponné avec le tampon ordinaire	$0{,}28 \pm 0{,}016$ $p < 0{,}02$	$1{,}03 \pm 0{,}06$
Tampon ordinaire	$0{,}40 \pm 0{,}03$	$0{,}98 \pm 0{,}11$
Solution isotonique de glucose tamponnée avec le tampon ordinaire	$0{,}77 \pm 0{,}03$ $p < 0{,}001$	$0{,}99 \pm 0{,}09$
Solution isotonique de glucose non tamponnée	$0{,}88 \pm 0{,}08$ $p < 0{,}001$	$1{,}15 \pm 0{,}03$

126 µg d'IgG-¹²⁵I ont été incubés avec 32,4 10⁸ érythrocytes. Le lavage des érythrocytes près l'incubation été fait avec les différentes solutions indiquées. La valeur de p indique une différence statistiquement significative par rapport à la valeur obtenue par lavage ec le tampon ordinaire. Valeurs $\bar{x} \pm SE$ nombre d'essais = 5.

Tableau I Fixation de l'IgG-¹²⁵I aux érythrocytes en fonction du nombre de lavages près incubation

Nombre de lavages	µg d'IgG-¹²⁵I fixés/culot + erre
2	$3{,}04 \pm 0{,}25$
3	$2{,}42 \pm 0{,}18$
4	$2{,}25 \pm 0{,}20$
5	$2{,}13 \pm 0{,}14$
6	$1{,}97 \pm 0{,}07$

100 µg d'IgG-¹²⁵I ont été incubés ec 32,4 10⁸ érythrocytes. Valeurs ± SE nombre d'essais = 5.

Comparaison de la fixation de l'IgG-¹²⁵I sur le verre et sur les érythrocytes L étude parallèle de la fixation de l IgG sur le verre et sur les érythrocytes permet de comparer l importance de l une de ces fixations par rapport à l autre Le tableau VI donne une comparaison de la fixation de l IgG-¹²⁵I aux érythrocytes et au verre rapportée à une unité de surface égale à celle d un érythrocyte (140 µm²) Cette comparaison a été faite avec différentes concentrations d érythrocytes. Le rapport de la surface du verre en contact avec l IgG ¹²⁵I sur celle d un

Tableau 17 Comparaison de la fixation d'IgG-^{125}I sur le verre et sur les érythrocytes

Erythrocytes incubés 10^{8}	Molécules d'IgG-^{125}I fixées par érythrocyte 10^{3}	Molécules d'IgG-^{125}I fixées par 140 μm^2 de verre 10^{3}
81,0	0,66 ± 0,03	47 43 ± 6,28
8,1	4,72 ± 0,14	55,56 ± 4,82
0,81	125,67 ± 9,0	130,0 ± 6,07

400 µg d'IgG-^{125}I ont été incubés avec un nombre variable d'érythrocytes. Les µg sont convertis en molécules d'IgG en estimant le poids moléculaire de l'IgG à 1,5 10^{5} Le lavage des érythrocytes est fait avec une solution de glucose isotonique tamponnée avec le tampon ordinaire. Valeurs $\bar{x} \pm$ SE, nombre d'essais = 5.

Les valeurs dans la troisième colonne sont rapportées à une unité de surface égale à celle d'un érythrocyte (140 μm^2)

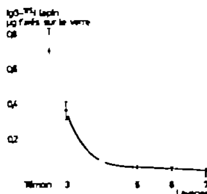


Fig. 6. Fixation de l'IgG-^{125}I sur le verre en fonction du nombre de lavages. L'incubation met en présence 482 µg d'IgG-^{125}I avec 8,1 10^{8} érythrocytes. Chaque point représente la moyenne de 2 valeurs ± l'erreur moyenne de la moyenne ($\bar{x}$ ± SE). Le témoin indique la valeur de la fixation sur le verre sans érythrocyte après le 3^{e} lavage.

érythrocyte est de 2,6 10^{6} Le nombre de molécules d'IgG-^{125}I fixées sur le verre est divisé par 2,6 10^{6} pour obtenir le nombre de molé cules d'IgG fixées sur une surface de verre égale à celle d un érythrocyte. Ces résultats indiquent que la fixation de l'IgG-^{125}I sur le verre

augmente à mesure que le nombre d érythrocytes incubés diminue cette augmentation est toutefois moins prononcée que pour la fixation cellulaire de l IgG [131]I En présence d un nombre minimum d érythrocytes, la fixation de l IgG [131]I au verre et aux érythrocytes est égale pour une même unité de surface (140 μm^2) L influence du nombre de lavages sur la fixation de l IgG-[131]I au verre est aussi étudiée (fig 6) Un témoin, en l absence d érythrocytes, indique la valeur de la fixation de l IgG [131]I sur le verre après 3 lavages On voit que la fixation de l IgG-[131]I sur le verre diminue de façon exponentielle avec le nombre de lavages. Différentes expériences étudiant la fixation de l IgG-[131]I sur le verre montrent qu elle est proportionnelle à la surface du verre et qu elle dépend de la nature du verre. La fixation de l IgG-[131]I sur divers tubes de matière plastique est plus importante que celle mesurée sur le verre.

Discussion

Nos résultats montrent que, dans les conditions étudiées, la fixation non spécifique d IgG aux érythrocytes varie de 0 5 à 1 de la quantité totale d IgG incubée. Les nombreux constituants cytophiles du sérum peuvent, en contaminant l IgG majorer le pourcentage de fixation non spécifique obtenu par différents auteurs [13 23] Dans ce travail les critères de pureté de l IgG permettent d exclure la présence de contaminants sériques cytophiles et d affirmer que la radioactivité mesurée représente l IgG D après les travaux de Frommel et Gros [11] la présence d agrégats augmente aussi la fixation cellulaire non spécifique de l IgG La formation d agrégats étant un processus continu la présence d agrégats ne peut être éliminée par les méthodes habituelles dans les préparations d IgG utilisées. On sait, par ailleurs, que les IgG agrégées à 63 C se fixent près de 15 fois plus sur les érythrocytes que les IgG non agrégées [15] La persistance de la radioactivité dans le culot, après lavage, prouve que l IgG est fixée aux érythrocytes. La fixation de l IgG se fait sur la membrane cellulaire et une pénétration de l IgG dans la cellule est exclue puisque, après centrifugation des érythrocytes hémolysés, la radioactivité du surnageant est égale à celle du back-ground. La réaction de fixation de l IgG aux érythrocytes est très rapide et n est pas modifiée par une incubation prolongée En diminuant le nombre d érythrocytes in-

cubés en présence d une quantité constante d IgG la quantité d'IgG fixée par érythrocyte augmente Ceci indique que la surface de l érythrocyte ne limite pas la fixation non spécifique de l IgG comme c est le cas pour la fixation spécifique. D autre part, lors de l adjonction de quantités croissantes d IgG aux érythrocytes, la fixation cellulaire de l'IgG augmente et la surface érythrocytaire n est pas saturée par une quantité bien définie d IgG bien que le pourcentage de fixation cellulaire diminue progressivement. Ceci suggère qu une association de molécules d IgG entre elles pourrait favoriser la fixation cellulaire non spécifique de l IgG De même les propriétés cytophiles des complexes antigènes-anticorps solubles ont été utilisées pour la sensibilisation passive de différentes cellules [28] Selon ces auteurs, les complexes antigènes-anticorps stabiliseraient la fixation cellulaire en diminuant la dissociation spontanée des molécules à la surface de la membrane l association des molécules d IgG entre elles facilitée par une forte concentration d IgG pourrait agir de façon semblable lors de la fixation non spécifique.

La fixation non spécifique de l IgG aux érythrocytes et au verre diminue lorsque le *pH* du milieu d'incubation augmente de 5 5 à 7 8. Ce fait indique que les forces électrostatiques interviennent dans la fixation non spécifique. Nos résultats sont en accord avec ceux obtenus par Mollison et coll [21] alors que Grob et coll [13] décrivent un optimum de fixation non spécifique à un pH de 7 6 comme pour la fixation spécifique [19]

Un accroissement de la *force ionique* du milieu d'incubation ou du tampon de lavage après l'incubation produit une diminution de la fixation non spécifique de l'IgG Le même phénomène a été décrit pour la fixation spécifique [19] Dans un milieu de faible force ionique, l'association des molécules de protéine entre elles est plus grande, ce qui permettrait d expliquer en partie l effet de la force ionique sur la fixation cellulaire de l IgG D après les travaux de Pollack et coll [24] l abaissement de la force ionique influence aussi la membrane érythrocytaire en diminuant son potentiel électronégatif «zeta» ce qui favorise la fixation spécifique des hémagglutinines.

L augmentation de la *température* du milieu d incubation diminue la quantité d IgG fixée non spécifiquement aux érythrocytes. Ces résultats sont en accord avec les travaux de Spuzic et coll. [26] La diminution de la fixation non spécifique de l IgG à 37°C montre que les forces qui maintiennent l IgG à la surface de l érythrocyte sont faibles et

diminuent lorsque l'énergie cinétique des molécules d'IgG s'accroît. Le fait que les IgG fixées non spécifiquement aux érythrocytes ou au verre puissent être éluées en grande partie par des lavages répétés, indique aussi que l'IgG fixée non spécifiquement est moins fortement liée à la membrane érythrocytaire qu'elle ne l'est lors de la fixation spécifique. La variation de la fixation de l'IgG en fonction du nombre d'érythrocytes et l'existence d'une compétition entre la surface du verre et celle de l'érythrocyte indiquent que la fixation non spécifique de l'IgG a lieu sur la surface cellulaire ou non cellulaire mise à disposition.

Nos remerciements vont à feu le Dr T. Webb pour ses conseils de valeur ainsi qu'au Dr M. Waldenström. Nous remercions aussi Mesdemoiselles H. Wili et B. Filloux pour leur assistance technique précieuse.

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Résumé

La fixation non spécifique des IgG aux érythrocytes est étudiée *in vitro* chez le lapin en modifiant successivement la concentration d'érythrocytes, d'IgG ainsi que le mode d'incubation. Les IgG ne se fixent pas seulement sur les érythrocytes, mais encore sur des supports non cellulaires. Le mécanisme de fixation de l'IgG sur le verre semble différer de celui observé sur les érythrocytes. Une saturation de la surface érythrocytaire n'est atteinte qu'avec des quantités très importantes d'IgG. En présence de concentrations croissantes d'IgG, le pourcentage de fixation diminue mais la quantité absolue fixée par cellule augmente progressivement, probablement en raison d'un phénomène d'association des molécules d'IgG.

Summary

The non specific *in vitro* fixation of IgG to red blood cells has been studied by varying successively the concentrations of erythrocytes, IgG and also the mode of incubation. The results show that IgG fixes not only to red cells but also to any supporting material. In the case of glass the mode of fixation appears to differ from that observed in erythrocytes. A slow saturation of the red blood cell surface is approached only with large quantities of IgG. Although the percentage of fixation diminishes with increasing amounts of IgG, the absolute amount fixed per cell increases progressively probably due to association of IgG molecules.

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Adresse des auteurs: Dr Dominique des Gouttes et Prof. Dr H. Isliker, Institut de Biochimie, Université de Lausanne, 1000 Lausanne (Suisse)

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Haemoglobin L Persian Gulf: α57 (E6) Glycine → Arginine

S. RAHBAR, JUDITH L. KINDERLERER and H. LEHMANN

Department of Immunology, University of Tehran, and Medical Research Council Abnormal Haemoglobin Unit, Department of Biochemistry, University of Cambridge, Cambridge

The haemoglobins of 3,200 patients at Tehran University Hospitals were screened for variants by electrophoresis. A number of known and unknown variants of haemoglobin A were found and we wish to report the identification of one of these. Its electrophoretic properties were identical to those of haemoglobin L [1]. No anaemia was detected, the morphology of the red cells was normal, there were no inclusion bodies, no evidence of increased methaemoglobin formation nor was any decrease in haem binding found. This is of some relevance as the *substitution was shown to be next to the distal haem linked histidine* of the α-chain.

As the amino acid substitution in haemoglobin L has not yet been identified it is quite possible that the new variant and haemoglobin L previously described [1] in a Hindu Punjabi family and in 3 Gujarati speaking Lohana families in Bombay [15] are not the same. The present haemoglobin was found in a woman from an area bordering the Persian Gulf, and we have called it haemoglobin L Persian Gulf. Unfortunately only one other member of the family, a son, was available for screening and he did not have the variant.

On electrophoresis at alkaline pH on paper or starch, the variant moved slightly faster than haemoglobin S but more slowly than haemoglobin G (fig. 1). On agar gel electrophoresis at pH 6 [12] there was no clear separation from haemoglobin A. On chromatography at pH 6 the variant moved between haemoglobins S and C [8]. On starch gel electrophoresis [11] a second haemoglobin A_2 was noted, and this together with the fact that the proportion of the abnormal variant, though not unstable, was only 18 %, suggested that the variant

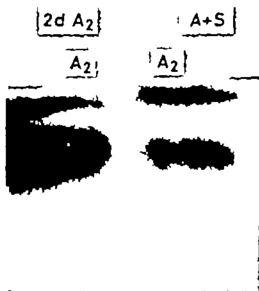


Fig. 1. Paper electrophoresis (pH 8.6). Left: Hb A and Hb L; right: Hb A and Hb S. Hb L moves slightly faster than Hb S towards the positive pole. The position of the Hb A_2 fractions is indicated. — line of application.

was abnormal in the α-chain [10]. This was confirmed by examining the whole haemolysate by electrophoresis after treatment with para mercuribenzoate [3], after electrophoresis of the isolated abnormal variant in 6 M urea [4] and by its hybridisation with canine haemoglobin [6].

Methods

The electrophoretic studies and the isolation of the variant, its digestion with trypsin, the preparation of fingerprints (peptide chromatograms) and the staining of the peptides were all carried out according to the techniques summarised by Sick et al. [13], except that in addition to paper electrophoresis, ion exchange chromatography on DEAE Sephadex was used for the isolation of this variant [7].

Results

Figure 2 shows a fingerprint of the whole globin in which two differences from normal globin were observed. The first occurred in

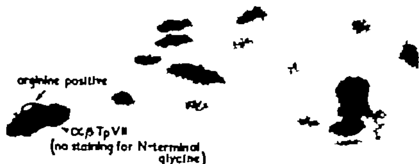


Fig 2. Fingerprint (peptide chromatogram) of the isolated Hb L Persian Gulf.

Table I. The amino acid residues and their sequential numbers in the tryptic peptides (Tp) and β VII of human haemoglobin

Chain	Number in helical sequence	E6	E7	E8	E9
	Number in chain	57	58	59	60
	Residue of	glycine	histidine	glycine	lysine
β	Number in chain	62	63	64	65
β	Residue of	alanine	histidine	glycine	lysine

the overlap of two peptides, αTpVII containing the amino acid residues α57–60 and β62–66 (α and β E6–E10) which failed to show the transient yellow colour on treatment with ninhydrin which normally indicates the presence of the N-terminal glycine residue in αTpVII (Gly His-Gly Lys) The two peptides include the distal haem linked histidine residues of the α- and β-chain respectively (table I) The second abnormality was a positive staining for arginine observed slightly behind and above the lysine of α β TpVIII (α61 Lys and β66 Lys) To eliminate the peptides from the β-chain for subsequent determination of the change the and β-chains were separated [5] and aminoethylated [9] Figure 3 shows the fingerprint of the amino-



Fig 3. Fingerprint (peptide chromatogram) of the aminoethylated α-chain of Hb L. Free arginine (TpVII) and lysine (TpVIII) were found together with peptide containing histidine, glycine and lysine (TpVIIb, see table II)

Table II Amino acid analysis of TpVIIb in figure 3

Amino acid	μ mol	Residues found	Residues expected in haemoglobin A
Histidine	0.00785	0.9	1
Glycine	0.01063	1.2	2
Lysine	0.00885	1.0	1
Total		3.1	4

ethylated α-chain and gives the position of three ninhydrin positive spots, which on amino acid analysis were shown to consist of free lysine free arginine, and of a peptide containing histidine glycine and lysine respectively The electrophoretic mobility of haemoglobin L indicates the acquisition of one positive charge per α-chain The finding of free arginine locates the substitution at the N terminal of a tryptic peptide of the α-chain by arginine or substitution by lysine or arginine of the penultimate residue of a tryptic peptide with a carboxy C-terminal arginine There are only five positions in the

α-chain where a single point mutation could give rise to free arginine after tryptic digestion and where this would result in a haemoglobin which has one additional positive charge per α-chain These are the N-terminal residues of threonine in αTpII and αTpVI methionine in αTpV glycine in αTpVII and leucine in αTpXII Of these αTpII V and VI are clearly present in their expected positions, and where applicable, with the expected staining reactions in the fingerprint of the whole globin (fig 2) Peptide αTpXII is part of the insoluble core and cannot be demonstrated in the fingerprint of the whole globin, but αTpXIIa is present, and gives the expected reaction for histidine and sulphur in the fingerprint of the aminoethylated chain (fig 3)

In the fingerprint of the whole globin, αTpVII, however failed to show the expected yellow reaction on treatment with ninhydrin which indicated the absence of an N terminal glycine residue in position α57 In the fingerprint of the isolated α-chain the yellow reaction with ninhydrin for the N-terminal glycine residue of αTpVII was not observed, and αTpVII was not in the expected position but had a slight decrease in positive charge and a lower chromatographic mobility compared with αTpVII in haemoglobin A.

This peptide was isolated by electrophoresis at pH 2 from the aminoethylated α-chain On amino acid analysis one residue each of glycine, histidine and lysine was found (table II) As this peptide is formed after hydrolysis by trypsin from the α-chain, the lysine residue would be C-terminal and the glycine could not by N-terminal due to the failure to give the yellow colour with ninhydrin, thus the N-terminal residue would be histidine at α58. This peptide is therefore αTpVIIb accounting for residue α58–60 the free arginine in this case would arise from a substitution of α57 (E6) glycine to arginine. From the sequence of αTpVII and VI given in table III it can be

Table III. Sequence of TpVI and TpVII from haemoglobin L Persian Gulf

Sequential numbers							
				TpVII ⟵⟶			
41	42	55	56	57	58	59	60
Thr	Tyr	*Val*–	*Lys*–	*Arg*–	*His*–	*Gly*–	*Lys*

↑ after Lys 56, ↑ after Arg 57, ↑ after Lys 60; VIIa = Arg 57; VIIb = His 58–Lys 60

↑ = position of hydrolysis by trypsin.

seen that tryptic hydrolysis would take place at the lysine residue α56 giving rise to αTpVI and the arginine residue α57 giving free arginine of αTpVIIa and at the lysine residue α60 giving αTpVIIb. Thus haemoglobin L Persian Gulf can be defined as α57 (E6) glycine → arginine.

Discussion

The glycine residue E6 is next to the distal haem linked histidine residue α58 (E7) and it is perhaps surprising that a change from glycine to arginine at this position should not cause some abnormality at least in the haem binding or in the tendency to methaemoglobin formation. However it must be noted that this residue varies greatly in known globins and indeed haemoglobin Norfolk with substitution α57 glycine to aspartic acid at the same position [2] does not show any pathological effect. In this connection we have re-examined haemoglobin Norfolk and found its susceptibility to oxidation to be normal.

It will be of interest to find out whether the haemoglobin L found sporadically in Indians is identical with haemoglobin L Persian Gulf. The variant observed in Bombay has been investigated [14] but these preliminary findings do not support the suggestion that these haemoglobins are the same.

Summary

A new haemoglobin has been identified from an Iranian woman. It has been designated haemoglobin L Persian Gulf, α57 (E6) glycine → arginine. This variant of adult haemoglobin was not associated with any pathological conditions.

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Authors' addresses: Dr S. Rahbar, Department of Immunology, University of Tehran, *Tehran* (Iran); Mrs. Judith L. Kinderlerer and Prof. Dr H. Lehmann, MRC Abnormal Haemoglobin Unit, University of Cambridge, Department of Biochemistry, Tennis Court Road, *Cambridge* (England)

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Research Institute of Child Health, Hacettepe Children's Hospital, Ankara

Haptoglobin in Thalassemia

B. YAMAK and S. ÖZSOYLU

The term haptoglobin (Hp) was introduced by POLONOVSKI and JAYLE to describe a group of serum glycoproteins specifically capable of binding hemoglobin *in vitro* and *in vivo* by forming complexes with it [1]. *These haptoglobin hemoglobin (Hp-Hb) complexes in vivo* are taken up by the reticuloendothelial cells from the circulation where Hb degradation occurs. If intra vascular hemolysis increases, plasma Hp concentration decreases [2, 3]. By starch-gel electrophoresis, different kinds of Hp have been shown in the α_2-fraction of the serum which are inherited genetically [4] and there is substantial evidence that its type is somewhat related to D chromosomes [5].

The absence of this protein is well documented in sickle-cell anemia [6], but Hp in thalassemia has been determined in only a few cases [8–10]. For this reason, we looked for Hp in thalassemia major and minor.

Materials and Methods

In this study 35 cases of β-thalassemia major are presented in which the diagnosis was made clear by early developing hypochromic anemia, marked hepatosplenomegaly, increased serum iron and fetal hemoglobin, decreased erythrocyte osmotic resistance, absence of abnormal hemoglobins, and the presence of nucleated red blood cells (NRBC) on the peripheral smear. The patients' ages ranged from 6 months to 13 years and only 13 of them were girls. Forty-two β-thalassemia trait cases were found in either the parents (32) or the siblings (10) of the patients.

In all the patients, hemoglobin (Hb), hematocrit (Hct), white blood cells, reticulocyte, peripheral smear, sickling and Coombs test were determined by the standard methods. In all the homozygous cases bone marrow was also examined. The alkali-resistant Hb levels were

determined by the method of SINGER *et al.* [11]. Starch-gel (pH 8.6) and agar-gel (pH 6.45) electrophoresis were performed by the techniques of SMITHIES [12] and ROBINSON *et al.* [13] respectively. Red blood cell osmotic fragility was done according to DACIE's methods [14] following 24 hours incubation at 37°C. For the detection of Hp, serum samples obtained just prior to transfusion were subjected to horizontal starch-gel electrophoresis at pH 8.6 (tris-citric acid) [12]. Before electrophoresis, sufficient Hb was added to the serum to saturate completely any Hp present. One half of each resulting gel pattern was stained with amido-black for the detection of protein bounds, and the other half with benzidine stain to indicate the Hp-Hb complexes. These were compared with the serum of normal subjects. Three of the patients with thalassemia major had infection (cases 3, 26, 33) at the time when blood samples were drawn for Hp determination.

Results

The laboratory findings of the homozygous patients are shown in table I. In 23 patients, minimal Hb A component was observed by agar-gel electrophoresis. In 11 of these 23 cases Hb A could also be shown by starch-gel electrophoresis. Hb A_2 was not measured but estimated according to normal control Hb separation. Hp could not be shown in 26 of the 35 thalassemia major cases (74%). It was present in the normal amount in 7 of the cases (20%) and barely present in 2. In general, the spleen was more markedly enlarged than the liver. Splenectomy was performed in 7 cases when the transfusion intervals had become less than a month, causing some improvement. Five out of 35 patients with thalassemia major had previously had jaundice. In 2 cases (14 and 17) where it occurred in the neonatal period, it was accepted as a physiologic jaundice; in case 21 jaundice was noticed at 6 months of age for a month's duration, and in 2 (8 and 32) it occurred at 7 and 14 years of age respectively, for which serum hepatitis was the most likely diagnosis.

Hp, Hb electrophoresis and Hb F values in patients with thalassemia trait are summarized in table II. In all but 7 cases Hp was found. Four of the *ahaptoglobinemic thalassemia minor cases* belonged to one family and 2 to another family, which indicates the possibility of familial ahaptoglobinemia.

Discussion

Hp is synthesized by the liver and taken up by the reticuloendothelial cells when in combination with Hb *in vivo*. This protein in-

Table I. Laboratory findings of the thalassemia major cases

Case No.	Age Sex	Hp	Hb g%	Ret %	Hb F %	Hb electrophoresis Starch gel A_2			Agar gel F	A
						A_2	F	A	F	A
1	12 F	A	4.93	2.2	62	+	+	–	+	–
2	3 M	1 2	3.50	2.8	47.3	+	+	–	+	+
3	8/12 M	1 2	6.63	2.2	84	+	+	–	+	+
4	2 M	1 2	4.07	0.6	32	+	+	+	+	+
5	11/12 F	2 2	2.30	3.2	76	+	+	–	+	–
6	6 M	A	2.34	1.2	30	+	+	–	+	+
7	3.5 M	A	2.17	0.2	69.4	–	+	–	+	–
8	14 F	A	7.34	1 4	57 4	+	+	–		
9	4.5 M	A	3.85	11	12	+	+	+	+	+
10	3.5 F	A	4.80	0.4	19	+	+	+	+	+
11	5 M	A	6,20	0,8	19	+	+	+	+	+
12	13 M	A	7.05	3.8	5.	+	+	+	+	+
13	8/12 F	A	4 71	5.4	74	+	+	–	+	+
14	2 M	A	3.13	1.6	81	+	+	–	+	–
15	7 M	2 2VS	8.60	3.6	87	+	+	–	+	–
16	1.5 M	A	7.82	3	88	+	+	–	+	+
17	2.5 M	A	6.20	3.6	73	+	+	–	+	–
18	4/12 M	A	7.25	3.2	80	+	+	+	+	+
19	1 F	1–2	5.15	9.2	70	–	+	–	+	–
20	6/12 F	1 2	5.54	4 4	82	–	+	–	+	+
21	4 M	A	2.37	0.6	39	+	+	+	+	+
22	1.5 M	2VS	4 71	3.2	68	+	+	–	+	+
23	1.5 F	A	5.16	4.8	95	+	+	–	+	–
4	4 M	A	2.98	2.6	54	+	+	–		
25	3 F	A	2.85	7	92	+	+	–	+	–
26	6 1 F	A	7.05	5..	66	+	+	–	+	+
27	7/12 M	A	3.48	9.8	54 4	+	+	–	+	+
28	8 M	A	4.53	5.4	86	+	+	–	+	+
29	4.5 M	A	3.91	9.2	72	+	+	+	+	+
30	22 12 F	2 2	2.82	3	75	+	+	+	+	+
31	6 F	A	4.07	1.8	57	+	+	–	+	+
32	10 F	A	7.05	4	63	–	+	+	+	+
33	2 M	A	4.26	3.2	99	+	+	–	+	–
34	5 M	A	4.87	.6	60	+	+	+	+	+
35	20 1./M	A	3.75	13.2	50	+	+	–	+	+

VS Very slight, *A* [illegible]bsent, *Hb F* alkali-resistant hemoglobin, *Ret* reticulocytes
Splenectomy

creases in inflammatory neoplastic, degenerative processes, and following steroid treatment [15–17] Its level is found to be low in the new born period [18, 19] and in liver diseases [17 20] and to have the effect of estrogens [21] Although genetically determined absence of

Table II Laboratory findings of thalassemia trait cases

Family No.	Hp	Hb Electrophoresis					Hb F
		Starch gel			Agar gel		%
		A_1	F	A	F	A	
1 F	1 2	+	−	+	+	+	
2 M	2–2	+	−	+			
B-I	1 2	+	−	+	+	+	
B-II	1 2	+	−	+	+	+	
4 B-III	1 2	+	−	+	+	+	
B-IV	1 2	+	−	+	+	+	
6 F	1 2	+	−	+			
7 M	1 2	+	−	+	+	+	1 1
F	1 2	+	−	+	+	+	2.5
8 M	A	+	−	+	+	+	
S-I	A	−	−	+	+	+	
S-II	A	+	+	+	+	+	
B-I	A	+	−	+	+	+	
B-II	1 1 VS	−	−	+	+	+	
9 M	1 2	+	−	+	+	+	
F	2–2	+	−	+	+	+	
S	1 2	+	+	+	+	+	
10 S	1 1 VS	+	−	+	+	+	
14 M	A	+	−	+	+	+	6
F	A	+	−	+	+	+	4
15 M	1 2	+	−	+	+	+	6
F	1 2	+	−	+	+	+	7
16 M	1 2 Sl	+	−	+	+	+	6
F	1 1	+	−	+	+	+	4.5
17 M	1–2	+	−	+	+	+	8
F	1 2	+	−	+	+	+	8
21 M	1–2	+	−	+	+	+	4.5
22 M	1 2	+	−	+	+	+	1.5
F	2–2	+	−	+	+	+	2
23 M	1 2	+	−	+	+	+	5
F	1 2	+	−	+	+	+	3
26 M	2–2	+	−	+	+	+	2
27 M	1 2	+	−	+	+	+	1
F	2 2	+	−	+	+	+	2.2
29 M	A	+	−	+	+	+	5
F	1–2	+	−	+	+	+	2
30 M	2 2						
F	2 2						
31 M	1–2	+	+	+	+	+	3
F	1 2	+	−	+	+	+	5
35 M	1 2	+	−	+	+	+	2.8
F	1–2	+	−	+	+	+	3

B Brother *S* sister *M* mother *F* father *A* absent, *VS* very slight, *Sl* slight

this protein is well known [22 23] In general, decrease or absence of serum Hp has been observed in chronic or acquired types of hemolytic anemias in which the Hb turnover may be more than doubled, which indicates the severity of hemolysis [1 7]

Searches for Hp were made by JUNNARKAR [8] BADR EL DIN *et al.* [9] and MÜLLER EBERHARD *et al* [10] in 5 7 and 3 patients with thalassemia major respectively but none could be shown in any of them Neither did we find Hp in the serum of 74 , of our 35 thalassemia major cases. Nine cases where Hp was present in the serum appeared to be no different in any respect from the other cases. They were not on steroids, did not have jaundice and their transfusion re quirement was no less than that of the others. Their age NRBC, reticulocyte counts, the size of the liver and spleen Hb level, Hb F and the presence or absence of Hb A were no different from that in the rest of our patients.

Seven of the patients were splenectomized. In 2 of them, Hp was checked before and after splenectomy (4 and 10) In one Hp was shown before and after splenectomy but in the other it was not present in either determination. In one case Hp was determined only after splenectomy and in the rest only before splenectomy The 2 patients of BADR EL DIN *et al* [9] were splenectomized without any rise in the Hp level due to this procedure although it was stated that the Hb level was significantly raised in both. Since the hemolytic state per sists after splenectomy in thalassemia major this finding is not at all surprising When the rate of Hp utilization exceeds that of production or release of this protein, the Hp concentration falls to levels too low to be measured Because this glucoprotein was shown in one patient of BADR EL DIN *et al* [9] following infection, in 2 after milk injec tion and in our 9 cases, it is strongly suggested that ahaptoglobinemia in thalassemia is not due to the inability of the synthesis in general, but to increased consumption as is usual in hemolytic anemia

In 7 of the 42 thalassemia trait cases, Hp was absent. Six of these patients were from 2 families. A high incidence of ahaptoglobinemia is also reported in children with sickle cell trait [6 25] In our two families there were 3 and 5 patients with ahaptoglobinemia (including thalassemia major) Congenital ahaptoglobinemia may also be con sidered, since hemolysis is not a prominent finding in the thalassemia trait. If this holds true some correlation should be postulated between the thalassemia and Hp genes.

Hp was searched for in only 2 cases of thalassemia trait in the literature, and was not found in any which would also raise questions about the high incidence of ahaptoglobinemia in thalassemia [7 8]. In addition to COOLEY's anemia cases, we examined the serum of more than 200 subjects and with only one exception, whose family could not be studied, we were unable to find any ahaptoglobinemia without hemolytic anemia. Neither did ERDEM *et al.* [26] find any ahaptoglobinemia cases out of 300 people whose serum was examined in this country. That again might indicate that the high incidence of ahaptoglobinemia in thalassemia in this country may be related to the disease in some way.

Because thalassemia is a hemolytic anemia, the absence of Hp in the serum of most cases is not unexpected.

Summary

The type and presence of haptoglobins were determined in the serum of 35 and 42 patients with thalassemia major and minor respectively. Haptoglobins could not be shown in the starch gel electropherends in 74 and 17% of these groups of patients respectively. No correlation was found between the age, hemoglobin concentrations, transfusion frequency, size of spleen, liver, reticulocyte count, nucleated red blood cells and the presence or absence of Hb A electrophoretically in thalassemia major cases. It is pointed out that the incidence of ahaptoglobinemia among thalassemia trait cases seems to be more frequent.

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Authors' address: Dr [illegible] Yamak and Dr Sinan Özsoylu, Department of Haematology, Faculty of Medicine, Hacettepe University, Ankara (Turkey)

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Clinical Pathology Laboratories, Royal Victoria Hospital, Belfast

Evaluation of a Dual Radioisotope Urinary Excretion Test in the Diagnosis of Pernicious Anaemia

T. K. BELL and D. LEE

A rapid procedure for differentiating between patients with intrinsic factor deficiency and other causes of vitamin B_{12} malabsorption, which combined the two parts of the urinary excretion test introduced by SCHILLING [6] was reported by KATZ *et al.* [4]. These workers simultaneously administered $^{60}CoB_{12}$ bound to human gastric juice and free $^{57}CoB_{12}$ followed by a large flushing intramuscular dose of non-radioactive B_{12} and assayed the quantities of the two radioisotopes in the following 24 hour urine collection.

In a preliminary communication, BELL *et al.* [2] described a modification of this double isotope technique using $^{57}CoB_{12}$ bound to human gastric juice and free $^{58}CoB_{12}$, which replaced ^{60}Co resulting in a lower radiation dose to the patient. This dual radioisotope test for investigation of vitamin B_{12} absorption has been further evaluated and the results are now described.

Methods

Preparation of human gastric juice. Human gastric juice was obtained from normal subjects following maximal histamine stimulation [3] and, following the method of KATZ *et al.* [4], was immediately brought to pH 10 with 10% NaOH, and after 20 min neutralised to pH 7 with 1.0 N HCl. The juice was filtered to remove mucus and other deposits and the intrinsic factor content determined by the method of GOTTLIEB *et al.* [5]. Human gastric juice having concentration of active intrinsic factor less than 80% of the total B_{12} binding capacity was discarded. Bile-stained gastric juice was also rejected.

Preparation of radioactive B_{12} doses. Freeze dried ampoules of vitamin B_{12} labelled with ^{57}Co and with ^{58}Co each with specific activity of 1 $\mu c/\mu g$ B_{12} were obtained from the Radiochemical Centre, Amersham. Oral doses of $^{58}CoB_{12}$ and $^{57}CoB_{12}$ bound to human

gastric juice ($^{57}CoB_{12}HGJ$) each with concentrations of 0.25 µg B_{12} in 20 ml water were prepared as described previously [2]. Usually 12 radioactive doses, and appropriate standards, of the unbound and bound B_{12} were prepared at a time and these were stored at −15°C until required.

Dose administration. All subjects were fasted overnight. A dose of $^{58}CoB_{12}$ and dose of $^{57}CoB_{12}$ bound to human gastric juice were simultaneously administered orally followed immediately by an intramuscular injection of 1,000 µg of non-radioactive B_{12}. No food was allowed until 2 h later and all urine excreted in the 24 h after giving the doses was collected.

Measurement of the radioactive urine. Four hundred ml of the urine collection were assayed using an annular cell technique on a sodium iodide well type scintillation counter [1]. Since ^{57}Co emits γ rays with energies of 0.12 M V compared with ^{58}Co which emits γ rays with energies of 0.81 MeV the amount of each isotope in the total urine collection can be readily determined by altering the discriminator bias settings on an associated scaler and solving the simultaneous equations described by VEALL and VETTER [7], for the assay of liquid containing mixture of two radioisotopes.

Patients

One hundred patients have been included in the present retrospective study reflecting the use made of this dual isotope urinary excretion test by the clinical staff of this hospital, in the investigation of possible cases of B_{12} deficiency Patients in the following categories have been excluded (a) those who have had gastric surgery (b) those with renal failure, ( ) those with biochemical parameters supporting a diagnosis of 'malabsorption syndrome

The series includes 29 patients whose results were previously published [2].

The patients included in the pernicious anaemia group showed clinical picture consistent with the diagnosis supported by the finding of most of the following abnormalities: a macrocytic peripheral blood picture, megaloblastic bone marrow low serum B_{12} level, histamine fast achlorhydria or a satisfactory response to B_{12} therapy

Results

The pattern of results obtained is displayed in figure 1 and summarised in table I. Of the 62 control subjects only 4 gave $^{58}CoB_{12}$ excretions of less than 7% of the free $^{58}CoB_{12}$ dose. Only one control excreted less than 6% of this dose. All patients with pernicious anaemia (PA) excreted less than 6% of the $^{58}CoB_{12}$ dose. One patient with PA gave a $^{57}Co/^{58}Co$ ratio of 1 7 (which lies in the normal range of 0.8 to 1 8 for this series) and 2 patients gave a ratio of 1.9 In all other PA patients the $^{57}Co/^{58}Co$ ratio was greater than 2.0 A ratio of 35.7 was found in one PA patient but this result has not been included in the range and mean values for the ratio of ^{57}Co to ^{58}Co in the PA group.

Table I. Summary of results

Subjects		Percentage of dose excreted $^{57}CoB_{12}$ HGJ	Percentage of dose excreted $^{58}CoB_{12}$	Ratio $\frac{^{57}Co}{^{58}Co}$
62 Controls	mean	18.2 ± 6.4	16.2 ± 6.3	1.2 ± 0.2
	range	(5.7 33.0)	(4.0–32.1)	(0.8–1.8)
38 PA	mean	7.0 ± 2.9	2.2 ± 1.4	3.7 ± 2.0[1]
	range	(1.8–16.8)	(0.3–5.7)	(1.7–9.6)

Based on 37 results i.e. leaving out the high ratio of 35.7

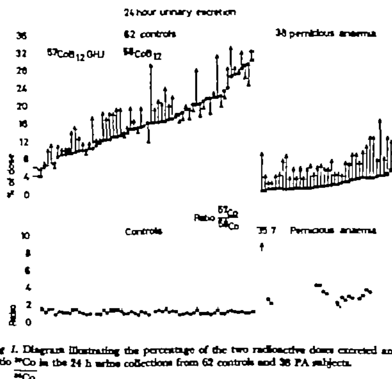


Fig. 1. Diagram illustrating the percentage of the two radioactive doses excreted and the ratio $\frac{^{57}Co}{^{58}Co}$ in the 24 h urine collections from 62 controls and 38 PA subjects.

Discussion

The present retrospective study has confirmed the accuracy and reliability of this test in the detection of deficiency of intrinsic factor in

Addisonian PA. In the previous study [2] all normals indicated a $^{57}Co/^{58}Co$ ratio of less than 1.6 while all those with PA, gave a ratio greater than 2.0. The results of studying a larger group of patients seem to indicate a slight overlap between the normal and pathological ranges, the range for patients with PA being 1.7 to 9.6, and for the control group 0.8 to 1.8. The result of the test agreed with the diagnosis made on other criteria in over 90% of the patients examined.

An attempt has been made to assess the possibility that high levels of intrinsic factor antibody present in serum and body fluids might inactivate the administered intrinsic factor and prevent its taking part in the absorption of B_{12}. Anti-intrinsic factor was sought in 36 patients in the present series including 12 subjects with PA and was demonstrated in 6 patients. All of these had PA, and had $^{57}Co/^{58}Co$ ratio in the PA range (values 2.1–5.5). Thus the presence of intrinsic factor antibody does not appear to interfere significantly with the test.

The simultaneous administration of $^{58}CoB_{12}$ and $^{57}CoB_{12}$ bound to intrinsic factor is advantageous to the patient, the clinician and the laboratory staff. Carried out in this way the test involves only one 24 hour collection of urine and the result can be available within 48 h of commencing the test. Recently the Radiochemical Centre, Amersham has set out to simplify the test further by presenting the bound and unbound radioactive B_{12} in the form of two capsules ready for administration thereby reducing the preparations for the test in the laboratory to a minimum. Preliminary studies with this preparation, which will soon be available commercially are promising.

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Summary

A retrospective study of the use of a urinary excretion test following the simultaneous oral administration of free $^{58}CoB_{12}$ and $^{57}CoB_{12}$ bound to human gastric juice in 100 patients is reported. The test provides an accurate evaluation of the intrinsic factor status in over 90% of the patients studied.

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Authors' address Dr T K. BELL and Dr D. LEE, Clinical Pathology Laboratories, Royal Victoria Hospital, *Belfast 12* (Northern Ireland).

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Cell Kinetics in Human Leukemia. Series Haematologica, vol. 1 No. 3. Munksgaard, *Copenhagen* 1968. 128 p.

Among the most interesting trends of modern hematology is certainly the study of the kinetics of leukopoiesis in normal and pathologic conditions. The availability of new investigatory methods such as the use of tritiated precursors of the nucleic acids synthesis and radioactive inorganic substances applied either through research *in vitro* or *in vivo*, has furnished data of great interest on the proliferative and maturative activities of the normal and leukemic leukocytes. It will be enough to emphasize that it has been definitely proven that in acute leukemia two clones of blastic cells do exist. One is proliferative and the other is temporarily or completely incapable of further divisions (sterile clone). This fully confirms the observation made in 1950–1963, using the stathmokinetic test, that the cell population of acute leukemia is endowed with low proliferative activity.

The last issue of the well-known 'Series Haematologica' deals exactly with the topic 'Cell Kinetics in Human Leukemia'. This publication is selling-out, complete set-up written by particularly qualified authors on the problems of leukocyte kinetics. There are contributed collections dedicated to the kinetics of chronic lymphocytic leukemia (L. M. Schiffer), chronic granulocytic leukemia (P. A. Chervenick and D. R. Boggs), and of acute leukemia (S. A. Killman). This chapter dedicated to kinetics of the leukemic blasts is especially ample, this field that has shown itself, more than any other, rich in results and new prospects. A stimulating contribution by S. A. Killman offers an interesting hypothesis on the mechanism with which the normal hemopoesis is suppressed by the abnormal blastic population in acute leukemia. These articles review and discuss the most recent acquirements and they serve as very useful aid both to those who study this subject and to those who want to keep up to date on the rapid progress being made in this area. All parts of the issue have quite numerous and accurate bibliographical references. This issue certainly contributes favorably to holding the prestige of 'Series Haematologica' high. A periodical that continues to be more and more successful.

G. Astaldi, *Tortona*

A. Galderisi, G. Riccio: *Lo studio dei cromosomi umani.* Capelli, 1966. 199 p. Preis L. 4500.

Dieses Buch gibt in 150 Druck- und Bildseiten eine Einführung in die menschliche Zytogenetik. Es enthält Kapitel über Chromosomenverteilung, Strukturanomalien, über klinische und zytogenetische Fakten der Trisomiesyndrome, Geschlechtsanomalien und Missbildungssyndrome, wie auch eine Besprechung der Chromosomenbefunde bei Neoplasien und hämatologischen Erkrankungen. Neben einleitenden Darstellungen, die stark vereinfacht sind, besteht dieses Buch vor allem aus einer weitreichenden Dokumentation der zytogenetischen Literatur bis 1964, wobei Wichtiges und Unwichtiges, Bewiesenes und Unbewiesenes nebeneinander stehen. Das Bildmaterial ist leider von schlechter Qualität. *Methodische Einzelheiten sind eingehend ausgeführt, jedoch sind die Originalarbeiten* nicht zitiert. Neue Techniken, wie Mikromethode oder Autoradiographie, fehlen. Da das Buch heute veraltet ist, sind naturgemäss noch Zusammenhänge aufgenommen, die bereits wieder fallengelassen worden sind. *Positiv zu erwähnen ist die Bibliographie von ungefähr* 1000 Autoren. *Leider ist sie nicht gegliedert* und bezieht sich z.T. nicht auf den Text, so dass erwähnte Stellen eines Autors unter Umständen nicht gefunden werden können. Druckfehler und Fehler substantieller Art dürften den Anfänger verwirren, so dass man sich fragt, wem man das Buch empfehlen soll. Der *italienischen* medizinischen Literatur wäre wohl mit der Übersetzung eines guten ausländischen Autors mehr gedient gewesen.

U. Lochmann, *Basel*

M. FRANÇON: **Einführung in die neueren Methoden der Lichtmikroskopie.** Übersetzt von LUDWIG ALBERT. G. Braun, Karlsruhe 1967. XI + 332 p., 340 fig. Preis: DM 56.–.

Die mikroskopischen Verfahren sind in den letzten Jahrzehnten um eine Reihe von sehr wesentlichen neuen Arbeitsmethoden bereichert worden. Diese neuen Mittel, wie Phasenkontrast- oder Interferenz-Mikroskope, sind nur erfolgreich anzuwenden, wenn die theoretischen Grundlagen bekannt sind. Zur Einführung in diese Grundlagen veröffentlichte M. FRANÇON 1961 einen Fortschrittsbericht «Progress in Microscopy» (Pergamon Press). Diese Monographie fand eine sehr gute Aufnahme, und dies veranlaßte 1967 eine ergänzte deutsche Übersetzung.

Das vorliegende Werk ist keine Anleitung für die praktische Mikroskopie; es vermittelt nur die Theorie. Da eigentlich alle neuen mikroskopischen Methoden nur mittels wellenoptischer Betrachtungen erfaßbar sind, so bringt das einleitende Kapitel in klarer, jede Umständlichkeit vermeidender Weise die Grundlagen der Bildentstehung im Mikroskop. Damit ein solches Bild – besonders von sehr kleinen Objekten – richtig interpretiert werden kann, muß absolute Klarheit vorhanden sein über das Wesen eines derartigen Bildes und ebenso auch über die Faktoren (Beleuchtungsprinzip, Wellenlänge des Lichtes, Optikeigenschaften), welche die Bildqualität beeinflussen oder gar verfälschen können. Leider begegnet man in der Praxis immer wieder «Normalverbrauchern» mikroskopischer Geräte, die infolge ihres oft erschreckenden Nichtwissens um die Zusammenhänge ihre Apparaturen falsch einsetzen oder die Resultate falsch deuten.

Im zweiten Kapitel werden die Grundlagen und die Anwendungsmöglichkeiten der Phasenkontrastmikroskopie dargestellt. Da dieses Verfahren in seiner landläufigen Anwendung schon lange bekannt ist und deshalb in den Standardwerken der Mikroskopliteratur gebührend berücksichtigt wird, hat sich der Autor hier etwas kürzer gefaßt. Hingegen geht er im folgenden dritten Abschnitt ausführlich auf die Interferenzmikroskopie ein. Nach einer kurzen, aber wiederum klar formulierten Einführung in das Prinzip werden die diversen technischen Ausführungen für die Interferenzmikroskopie vorgestellt. Das Kapitel schließt mit einem Hinweis auf die Empfindlichkeit dieser Methoden.

Unter dem Sammeltitel «Auflichtmikroskopie» bringt der nächste Teil alle Methoden, bei denen das Licht senkrecht oder leicht schräg durch das Objektiv auf das Präparat geführt wird. Nach der Erläuterung der verschiedenen Beleuchtungseinrichtungen und der kontraststeigernden Hilfsmittel folgt die Auflicht-Phasenkontrastmikroskopie, wobei wiederum die verschiedenen technischen Lösungen verglichen und erklärt werden. Ein weiterer Teil dieses Kapitels befaßt sich mit der schrägen Beleuchtung. Darunter ist nicht die gewöhnliche Epibeleuchtung mit der seitlich neben der Optik einstrahlenden Lichtquelle verstanden, sondern die Lichtschnittverfahren nach SCHMALTZ und nach MIRAU. Auch hier wird auf die praktischen Anwendungsmöglichkeiten hingewiesen; so können z. B. diese Methoden wertvolle Ergänzungen zu Phasenkontrast- oder Interferenz-Untersuchungen von Oberflächenstrukturen sein. FRANÇON bemerkt übrigens im Zusammenhang mit den einleitenden Betrachtungen über die Bildinterpretation, daß diese sehr erleichtert und gesichert werden kann, wenn die mikroskopische Untersuchung eines Präparats nach Möglichkeit mit verschiedenen Verfahren vorgenommen wird. Weitere Bestandteile dieses Kapitels bilden die Auflicht-Dunkelfeldmethode (mit dem Hinweis auf das Bestimmen von Neigungswinkeln) und das Auflicht-Interferenzverfahren. Wiederum werden die existierenden Geräte beschrieben und anschließend zeigt der Autor, welche Probleme damit gelöst werden können. Wie in den andern Teilen des Buches erfolgt das auch hier durch übersichtliche Schemata sowie durch Abbildungen von Geräten und Resultaten. Das Kapitel endet mit der Darstellung der Auflichtbeobachtung anisotroper Objekte im polarisierten Licht und mit einer Skizze über die Hochtemperaturmikroskopie.

In den folgenden Kapiteln werden verschiedene Meßmethoden und die dabei zu erzielende Genauigkeit (Kapitel 5) besprochen, die Brechzahlbestimmung mit dem Zweistrahl-Interferenzmikroskop (Kapitel 6), die Möglichkeiten des Polarisations-Interferenz-

mikroskops (Kapitel 7) sowie im Kapitel 8 Dicken-, Rauhigkeits- bzw. Winkelbestimmungen. Biologisch interessieren dürfte in diesem Abschnitt die interferenzmikroskopische Bestimmung des Trockensubstanzgehaltes von lebenden Zellen.

Während in den zuvor kurz referierten Kapiteln 5–8 der Mediziner nur verhältnismäßig wenig Anwendungsmöglichkeiten findet, dürfte der nächste Abschnitt über die mikroskopischen Methoden mit infraroter und ultravioletter Strahlung wieder von erheblicher praktischer Bedeutung sein. Auf die Skizzierung der technischen Voraussetzungen, wie Spezialoptik oder Kondensor für UV folgen die Aufzeichnungs- und Beobachtungsmittel, in erster Linie der Bildwandler, der Leuchtschirm und die Fernsehkamera. Den Abschluss dieses Kapitels bildet die Erläuterung von verschiedenen Möglichkeiten der Fluoreszenzmikroskopie; besonders interessieren könnte hier der Hinweis auf die Phasenkontrast-Fluoreszenzverfahren.

Wieder mehr den Chemiker und den Physiker werden die im zehnten Kapitel aufgeführten Gebiete der Mikrospektrometrie und der Mikrospektrophotometrie ansprechen. Von allgemeinem Interesse sind dafür die im nächsten Teil erklärten Methoden des Flying Spot (Scanning)- und des Laser-Mikroskops. Das Buch verfügt zwar über ein sehr umfangreiches Verzeichnis von einschlägiger Literatur, doch wäre hier zur Einführung in die sicher noch längst nicht ausgeschöpften Möglichkeiten eine ausführlichere Darstellung erwünscht. Ebenso wäre auch eine reichere Bebilderung des Kapitels am Platze gewesen, um die Resultate dieser wirklich neuartigen Verfahren zu dokumentieren.

Das letzte Kapitel ist mikroskopischen Untersuchungsmethoden in der Chemie reserviert; selbstverständlich findet man darunter auch Anwendungsmöglichkeiten für die physiologische Chemie. Den Schluss des Buches bilden das 46 Seiten umfassende, kapitelweise gegliederte Literaturverzeichnis sowie die üblichen Register.

In verdienstvoller Arbeit haben der Autor und ebenso der ausgezeichnete Übersetzer Ludwig Albert ein Werk geschaffen, das vorzüglich zur Förderung des Wissens um die verschiedenen neuen Verfahren geeignet ist. Selbstverständlich konnten unmöglich alle denkbaren Anwendungen in ausführlicher Weise beschrieben werden; es wäre sonst aus dem handlichen Buch ein unübersichtlicher Wälzer entstanden. Deshalb muss man sich beim Lesen immer wieder daran erinnern, dass das Werk eine Einführung darstellen soll. Diesen Zweck des Einführens hat François sicher erreicht, denn wie schon bemerkt, vermeidet er jeden abwegigen Ballast und beschränkt sich auf knappe Formulierung der Prinzipien. Gelegentlich wird allerdings mathematisches Denken vorausgesetzt. L. Jenny *Basel*

Actualités hématologiques. 2e Série. Publiées sous la direction de Jean Bernard. Masson, Paris 1968. 258 p., 55 fig., 60 Tab. Preis 50 NF

Die zweite Serie der «Actualités hématologiques», die wiederum unter der Leitung von Prof. Jean Bernard und unter Mitwirkung zahlreicher namhafter Kliniker und Forscher der französischen Hämatologie erschien, stellt sich dieselbe Aufgabe wie die erste Serie, eine Übersicht zu geben über einige wichtige und aktuelle Gebiete der Hämatologie. Das erste vorwiegend klinisch orientierte Kapitel ist der akuten Leukämie und der Makroglobulinämie Waldenström gewidmet. Jean Bernard gibt aufgrund eines grossen eigenen Materials eine Übersicht über das Rubidomycin (Daunomycin), dessen therapeutischen Wert er vor allem bei unreifzelligen Myelosen sieht. Mit seiner hohen Aktivität und Toxizität eignet sich das Medikament vor allem für die Einleitung einer Behandlung. Auf die Möglichkeit einer Verlängerung der Remission unreifzelliger lymphatischer Leukämien durch die Methode der Reinduktion (vorwiegend kindliche Fälle!) weisen Jacquillat, Weil und Boiron hin. Weitere Abschnitte dieses Kapitels befassen sich mit der Meningosis leucaemica und mit symptomatischen therapeutischen Massnahmen bei akuten Leukämien. Aus dem Gebiet der Makroglobulinämien werden immunologische, zytologische, radiologische und therapeutische Gesichtspunkte dargestellt. Das zweite Kapitel befasst sich mit

der Zytologie der Lymphadenitis. Funktionelle Untersuchungen der Erythropoese, der Thrombozyten und der immunologischen Defekte bei malignen Lymphomen sind die Themen des dritten Kapitels. Ergebnisse neuerer hämatologischer Forschungen werden im letzten Kapitel zusammengefasst: Säugetierleukämien, Fusion und Hybridisierung von Säugetierzellen, Isotopenuntersuchungen über die Lymphozytenzirkulation bei chronischer lymphatischer Leukämie, Gewebsgruppen und schliesslich die Beeinflussung der Gerinnung durch Fibrinogenspaltprodukte.

Auch diese zweite Folge der «Actualités hématologiques» wird ihrem Titel gerecht, indem sie zahlreiche aktuelle Probleme zur Darstellung bringt. Dabei liegt das Hauptgewicht auf der klinischen Hämatologie, und es werden wie schon im ersten Band zahlreiche praktischen Fragen berücksichtigt. Das Buch vermag daher allen hämatologisch interessierten Ärzten wertvolle Informationen zu vermitteln. H. Lüdin, *Basel*

U. Willenbockel: **Zur Physiologie und Pathologie des Phosphatstoffwechsels unter besonderer Berücksichtigung der Phosphatverbindungen des kindlichen Blutes.** Mit Geleitwort von Prof. F. Linneweh. Beiheft 60 zu Arch. Kinderheilk. Ferd. Enke, Stuttgart 1969. XI + 109 p., 11 fig. 28 Tab. Preis DM 30.

In einer Einleitung, Physiologie des Phosphatstoffwechsels, gibt der Verfasser eine Übersicht über Phosphorverbindungen in der Nahrung, Phosphatresorption und Verteilung der Phosphate im Organismus, besonders im menschlichen Blut (Erythrozyten, Leukozyten, Thrombozyten und im Plasma). Hierauf wird die eigene Methodik geschildert. Der eigene Beitrag liegt hier vor allem in der säulenchromatographischen Trennung und Bestimmung freier Nukleotide und phosphathaltiger Metaboliten im Blut bei Kindern. Danach werden die Normalwerte der Ausscheidung der Phosphate im Stuhl und im Urin, der Phosphatbedarf und die Regulation des Phosphatstoffwechsels besprochen.

Etwa zwei Drittel der Monographie sind der Pathologie des Phosphatstoffwechsels gewidmet, wobei folgende Kapitel besprochen werden: Rachitis und Osteomalazie, Tetanie, Hyperkalzämie-Syndrome und Störung der Knochenmatrix sowie das Verhalten der Phosphatfraktionen bei anderen Krankheitszuständen. Bei den meisten der besprochenen Störungen berichtet der Verfasser über die mit seiner Methodik erhaltenen eigenen Resultate und kommentiert sie im Vergleich mit den schon bekannten Daten, insbesondere bei der Vitamin-D-Mangelrachitis, bei Zystinose, verschiedenen renalen Störungen, rachitogener Tetanie und Frühspasmophilie. M. Veit, *Basel*

C. J. D. Zarafonetis (editor): **Proceedings of the International Conference on Leukemia Lymphoma.** Lea & Febiger, Philadelphia 1968. 519 p. Price: US $ 15.00.

The conference held in Ann Arbor 1967 gives an excellent cross section about the present state and problems of leukemia and lymphomata. Several of the papers and panel discussions have as topic the viral etiology of leukemia (R. W. Mueller, A. C. Upton, W. R. Bryan, L. Dmochowski, J. T. Grace, W. R. Murphy, C. Zarafonetis and Sara E. Stewart). Very instructive is the presentation of Marcel Bessis on the ultrastructure of normal and leukemic granulocytes. W. S. Beck reviewing the biochemical properties of normal and leukemic leukocytes, is very sceptical whether assaying the leukocyte enzymes will contribute much to the knowledge of the nature of leukemia; in his opinion the basic problem remains the physiological mechanism which regulates leukopoesis. Of particular interest is the paper of Denis P. Burkitt on the African lymphoma, its epidemiological and therapeutic aspects. One section of the book contains papers and panel discussions of the present-day treatment methods in leukemia and lymphomata. These proceedings are of great interest for all haematologists, clinicians and experimental pathologists. G. Rosnow, *New York, NY*

L. G. Koss **Diagnostic Cytology and its Histopathologic Bases.** 2. Aufl. Pitman, London/ Lippincott, Philadelphia 1969. 633 S. mit 1200 Abb. Preis: £ 10.–.

Bereits die 1961 erschienene 1. Auflage dieses Buches war ein Standardbuch der diagnostischen Zytologie. Die jetzt nach 7 Jahren erschienene 2. Auflage ist gegenüber der ersten gründlich überarbeitet und vor allem im Kapitel über den weiblichen Genitaltrakt stark erweitert worden. Auch neuere Erkenntnisse der allgemeinen Zytologie wurden einbezogen.

Das Werk gliedert sich in zwei Hauptteile: «Allgemeine Zytologie» und «Diagnostische Zytologie der Organe». Im ersten Teil werden kurz, aber übersichtlich die Grundstrukturen der Säugerzelle dargestellt, wobei entsprechend der heutigen Bedeutung die Lysosomen allerdings relativ kurz berücksichtigt werden. Neben einer Darstellung der Aktivitäts- und Degenerationserscheinungen der Zelle, die für die Beurteilung von Ausstrichpräparaten besonders wichtig sind, werden auch andere pathologische Prozesse der Zellen etwa bei Entzündungen oder bei Hyperplasien und Metaplasien berücksichtigt. Die Teile über die Chromosomenstruktur, -dynamik und -pathologie sind mehr für den allgemein zytologisch interessierten Leser und weniger für den Zytodiagnostiker wichtig. Im zweiten Teil wird eine ausgezeichnete Darstellung der verschiedenen zytologischen Befunde im weiblichen Genitaltrakt, im Respirationssystem, im Nieren-Harnblasensystem und im ganzen intestinalen System gegeben. Ein besonderes Kapitel ist der Krebsfrühdiagnostik und ihrer Zuverlässigkeit gewidmet. Auch die Tumorzellen, die durch Aspirations-Biopsie gewonnen werden können, sowie die im Blut und Lymphe zirkulierenden Tumorzellen werden ausführlich beschrieben. Für den diagnostisch tätigen Pathologen ist der jeweilige Bezug der Histologie auf die Ausstrich-Zytologie von besonderem Wert. Durch diese Gegenüberstellung und den ausführlichen, gut gegliederten und leicht lesbaren Text unterscheidet sich dieses Buch wohltuend von anderen Büchern des gleichen Themas. Bei den Darstellungen der Vaginal-Ausstriche und der Zervix-Abstriche sind die Spiegelungsbefunde an der Portio und ihr histologischer und zytologischer Bezug berücksichtigt. Die Abhängigkeit des histologischen und zytologischen Bildes vom weiblichen Zyklus könnte bei Vergleich der anderen Kapitel etwas ausführlicher sein, da die Zyklusdiagnostik in der Praxis eine bevorzugte Rolle spielt. Bei den einzelnen Krankheiten werden die morphologischen Befunde sinnvoll durch Häufigkeitsstatistiken – z.T. unter *Einfluss der Therapie* – ergänzt. Praxisnah ist auch der Einbezug von Nebenbefunden, z.B. von Nematoden oder Pilzzellen im Sputum. Der Wert der Zytologie *für die klinische Diagnostik ist in mehreren Tabellen klar belegt, wobei auch* vielfach Verlaufsbeobachtungen mitgeteilt werden. Ein Abschnitt über die zytologischen Techniken enthält alle für die Praxis notwendigen Methoden einschl. *Hinweisen* für die zytologische Berichterstattung und für die sinnvolle *Organisation* zytodiagnostischer Laboratorien.

Das Buch gehört in die Hand aller Pathologen und Kliniker, die sich täglich mit der histologischen und zytologischen Tumordiagnostik befassen. Es enthält aber außerdem viele für die spezielle Pathologie der Tumoren wichtigen Einzelheiten, besonders in den Abschnitten über die Tumoren des weiblichen Genitaltraktes. Wer allerdings als Student oder als Anfänger die Zytodiagnostik lernen will, ist durch dieses Buch überfordert. Da die Reproduktionen der Mikrophotos kontrastarm sind, ersetzt das Buch bei der täglichen Arbeit nicht die vielfach in Buntdruck vorliegenden zytologischen Atlanten anderer Autoren. Es handelt sich also um ein Nachschlagewerk für den Fachmann. Ein gründlich bearbeitetes Sachregister ermöglicht ihm die Benutzung dieses sinnvoll gestalteten Buches.

E. Grundmann, *Wuppertal*

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Department of Haematology, St. Georg General Hospital, Hamburg, Data Processing Center, University of Hamburg, and Division of Medical Biochemistry, Institute of Physiological Chemistry of the University of Hamburg

Inter Relations between Iron Stores, General Factors and Intestinal Iron Absorption

K. HAUSMANN, R. KUSE, O. W. SONNENBERG, H. BARTELS and H. C. HEINRICH

Iron stores, rate of erythropoiesis, anaemia, hypoxaemia, transferrin saturation, gastric secretion, pancreatic secretion and dietary constituents have been reported to influence intestinal iron absorption [5].

For several decades routine measurement of ^{59}Fe retention after oral iron administration has been prevented by technical difficulties which have been overcome during the last years by ^{59}Fe whole body counting, but experience is still limited. The purpose of this paper is to summarize the results of studies on the correlation between the diagnostic criteria of iron metabolism reported preliminarily elsewhere [11–17, 21]. In 780 subjects including normal controls, patients with blood, liver and neoplastic diseases or infections three cytochemically different types of non haeme iron in bone marrow reticuloendothelial cells, sideroblasts, serum iron, total iron binding capacity (TIBC), transferrin saturation, haematocrit, haemoglobin, red blood cells, and the ^{59}Fe absorption whole body retention test (^{59}Fe AWBRT) (4 π large volume radioactivity detector with liquid organic scintillator) [16, 17] after administration of 0.558 mg Fe^{++} + 17.6 mg ascorbic acid in a strictly fasting state have been compared.

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Subjects

Totally 843 individuals have been examined, in part several times. The 780 subjects with comparable findings have been classified into the following groups: normal controls with normosiderosis (see definitions) 74 (males 36, females 38). Iron deficiency: prelatent iron deficiency 109, latent iron deficiency 27, iron deficiency anaemia 66, pregnancy with hyposiderosis 33. Gastrectomy: partial 19 (with hyposiderosis 12), total 3. Polycythaemia 34 (with hyposiderosis 28).

Different types of anaemia: hypoplastic anaemia 23, pure red cell anaemia 4, sideroachrestic anaemia 14 (with hyposiderosis due to treatment or bleeding 2, with prelatent or latent iron overload 8, with manifest iron-overload 4), untreated megaloblastic anaemia 10 (examined during reticulocytosis 7), haemolytic anaemia 11 (hyposiderosis 2), renal anaemia 1.

Malignant diseases: epithelial cancer 8 (hyposiderosis 1), Hodgkin's disease 72 (hyposiderosis 7), reticulum cell sarcoma or reticulosis 15 (hyposiderosis 2), acute leukemia in adults 18 (hyposiderosis 1), lymphocytic leukemia 47 (see table IV), myelocytic leukemia 17 (see table IV).

Chronic infections: 28 (hyposiderosis 1). Collagen diseases: 15 (hyposiderosis 5). Liver diseases: fatty liver 21 (hyposiderosis 1), liver cirrhosis 43 (hyposiderosis 13), chronic hepatitis 3, haemochromatosis 7. Miscellaneous: 47 (with hyposiderosis 10). Not included into the comparison: osteomyelofibrosis 10, without evaluable bone marrow iron 53.

Methods

Bone marrow smears with thick squash preparations of larger fragments aspirated from the sternum or the iliac crest were fixed in the vapour of neutralized formaldehyde 35% for 3 min. The slides were stained with 100 ml potassium ferrocyanide 1% + 1 ml hydrochloric acid 25% at room temperature for 15 min. About 50 oil immersion fields (microscopic magnification 10 × 40) of at least two preparations were examined by two independent observers. Cytochemical grading of the Prussian blue reaction was performed separately for the following three types of non haeme iron in bone marrow reticuloendothelial cells.

1. Diffuse cytoplasmatic iron staining of different intensity already described in the histochemical and cytochemical literature [see 4, 22, 24] was seen in isolated cells (fig. 1), cytoplasma buddings, and separated cell fragments of well preserved thinner areas of the squash preparations in specimens of subjects with normosiderosis and hypersiderosis. These elements could be followed easily into the thicker layers which appear spotted of diffuse blue to greenish. Cytochemical grading considered the colour scale and the number of positive cells in relation to the thickness of the area examined.

0	iron staining completely absent
(+)	traces, single faint blue cells in less than one quarter [(+)], two quarters (+) or three quarters (+)/+ of thick areas
1+	faint blue staining in nearly every appropriate field
2+ to 4+	increasing number of distinctly blue cells
5+ to 6+	numerous dark blue cells

2. Polymorphous haemosiderin particles were recorded 0 to 6+ according to their number and size in a similar way as described above. These aggregates occurred frequently in cells with and rarely in cells without the diffuse iron (fig 1). They could be seen already



Fig 1 Thin area of a bone marrow squash preparation (Prussian blue staining) with two iron containing macrophages surrounded by erythroblasts. Left cell: diffuse cytoplasmatic iron with two small haemosiderin particles. Right cell: haemosiderin particles of different size without diffuse iron. Single positive cell fragments.

in unstained preparations [28, 30] and seem to be identical with the "haemosiderin granules" of other authors [10, 23, 27, 30, 33]

3. Small uniform sized Prussian blue granules 0 to 6+ were observed after parenteral therapy with colloidal iron [4, 10] and in a more refractile state in haemochromatosis [4, 22], pernicious anaemia [31], and single cases of transfusional hypersiderosis in pure red cell anaemia after busulfan treatment of myelocytic leukaemia and single cases of sideroachrestic anaemia [own unpublished observations]

The report of LUNDIN *et al* [23] that histochemical iron grading in sections is equivalent to cytochemical grading in well preserved squash preparations is in accordance with own studies.

Sideroblasts were counted in 50–200 cells of the red cell series in thinner areas of the squash preparations. Only when identification of iron free immature erythroblasts was difficult slight counterstaining with aqueous eosin 0.1% (1 min) was performed.

Plasma iron was determined using bathophenanthroline [26] and total iron binding capacity according to CARAWAY [6] in fasting subjects. The venous haematocrit was measured in a microhaematocrit centrifuge. Haemoglobin was estimated as cyanhaemoglobin in an Eppendorf photometer using a commercially available standard. Erythrocytes were counted with the Coulter counter model A, B and F

Intestinal iron absorption was determined by means of the ^{59}Fe absorption whole body retention test (^{59}Fe-AWBRT) using a 4π large volume radioactivity detector with liquid organic scintillator [16]. An oral test dose of 0.558 mg $^{59}Fe^{++}$ (10 µMol = 0.05–0.20 µC) + 17.6 mg (= 100 µMol) L-(+) ascorbic acid was given in a strictly fasting state (see results and discussion). The retained radioactivity was measured 14 and 21 days later

Statistical analysis included conventional methods and non parametric tests, such as the Mann-Whitney U-Test for comparison of arithmetic means in different groups [29]. In correlation analysis computation of the Spearman rank correlation coefficients and the

product moment correlation coefficients gave corresponding results in smaller groups. In larger series the latter test was used only. The correlation matrices were subjected to the rotoplot program of factor analysis [19]. Details are reported elsewhere.

Definitions

In organizing data it has been found useful to classify the subjects examined into the following main groups defined by cytochemical grading of reticuloendothelial iron supplemented by the corresponding examination of liver iron, when iron overload is suspected.

Hyposiderosis	diffuse iron 0 to (+)/+ haemosiderin particles 0 to (+)
Normosiderosis	diffuse iron 1+ to 4+ haemosiderin particles (+) to 3+
Hypersiderosis	diffuse iron 5+ to 6+ haemosiderin particles 4+ to 6+

The term hyposiderosis includes every type and degree of symptomatic, co-morbid and co-normal iron deficiency independent of diagnostic criteria of iron metabolism other than exhausted iron stores. In the majority of cases iron deficiency can be subdivided by combination of different diagnostic ranges of at least three variables.

Prelatent iron deficiency	hyposiderosis, normal plasma iron (> 60 μg%), normal red cell indices haematocrit > 38%, haemoglobin > 12 g%, erythrocytes > 4.0 million/mm³
Latent iron deficiency	hyposiderosis, plasma iron < 60 μg, normal red cell indices
Manifest iron deficiency	hyposiderosis, plasma iron and red cell indices reduced

In corresponding way stages of reticuloendothelial and parenchymal iron overload are defined.

Prelatent iron overload	increased RES or parenchymal iron stores with normal plasma iron < 180 μg% or transferrin saturation < 60%, no tissue damage
Latent iron overload	hypersiderosis, increased plasma iron and transferrin saturation, no tissue damage
Manifest iron overload	hypersiderosis with increased plasma iron, transferrin saturation, and tissue damage

Results

During the initial period of this study measurement of ^{59}Fe retention in otherwise healthy young women and male blood donors with exhausted iron stores gave frequently far too low values in comparison with corresponding groups of in-patients. Such irregular effects could be considerably reduced in number when the subjects examined were asked repeatedly to remain completely fasting for 10 to 12 h before and 2 h after the administration of the test dose. Only some tap water was allowed. Iron loading before, during or after uncontrolled intake of little food or drinks diminished the arithmetic mean of intestinal

Table I. Effect of uncontrolled intake of food (or drinks) and iron therapy on intestinal iron absorption in the same subjects

	n	AM %	SD ± %	Range %
A. Prelatent and latent iron deficiency				
Fasting subjects	27	75.0	13.0	52.0– 97.0
Subjects not completely fasting	27	32.9	16.6	6.2 57.0
B. Different stages of iron deficiency Oral iron therapy 6.0–12.0 g Fe⁺				
Before therapy	18	91 7	9.3	71.0–100.0
After therapy (diffuse iron ≥ 1 +)	18	32.0	9.3	8.5– 48.0
C. Iron deficiency anaemia Parenteral iron therapy 1.0–3.5 g Fe⁺⁺⁺				
Before therapy	16	88.2	5.8	80.1 98.2
After therapy (see text)	16	19 1	11.2	6.0– 46.8
Relapse despite of residual bodies 1 + to 4 + (only in part the same subjects)	15	88.2	10.5	63.5–100.0
D. Prelatent and latent iron deficiency Parenteral iron therapy				
Before therapy	13	72.4	12.9	48 6– 92.5
After therapy	13	19 4	10.5	7.5– 48.1

number of subjects examined, *AM* arithmetic mean of iron absorption values, *SD* standard deviation.

iron absorption by the factor 2.3 in the same individuals with prelatent and latent iron deficiency serving as their own controls when they were strictly fasting during the critical period (table I A P < 0 01) Similar observations were made in few subjects with different diseases.

In single cases with severe iron deficiency anaemia excluded from this study heavy blood losses after the administration of the test dose reduced the ⁵⁹Fe retention by about 20–30% as estimated by the subsequent determination of the ⁵⁹Fe whole body turnover rate.

The first group of table II (n = 780) consisted of subjects with comparable results. The second series (n = 646 *table II table III B,* fig 2) excluded all groups totally in which irregular results were observed (see also table IV)

A close negative correlation has been computed between the diffuse Prussian blue reaction of bone marrow RE cells and the ⁵⁹Fe AWBRT

Table II Correlations

Correlations between	All cases n = 780	± CI	Without dysregulation n = 646	± CI
Diff. F – ^{59}F-AWBRT	−0.76	0.04	−0.88	0.03
Diff. Fe – haemosiderin	+0.64	0.06	+0.65	0.07
Diff. F – TIBC	−0.60	0.06	−0.66	0.06
Diff. Fe – UIBC	−0.64	0.06	−0.68	0.06
Diff. Fe – plasma iron	+0.35	0.06	+0.35	0.08
Diff. F – sideroblasts	+0.50	0.07	+0.50	0.08
^{59}Fe-AWBRT – haemosiderin	−0.49	0.07	−0.57	0.08
^{59}Fe-AWBRT – TIBC	+0.59	0.06	+0.66	0.06
^{59}F-AWBRT – UIBC	+0.57	0.07	+0.67	0.06
^{59}Fe-AWBRT – sideroblasts	−0.35	0.09	−0.45	0.09
^{59}Fe-AWBRT – plasma iron	−0.24		−0.32	0.10
^{59}Fe-AWBRT – haemoglobin	+0.07		+0.06	
Plasma iron – sideroblasts	+0.53	0.07	+0.52	0.08
Transferrin-sat. – sideroblasts	+0.60	0.06	+0.60	0.07

r = correlation coefficient, CI = confidence interval of the correlation coefficient ($P < 0.01$) Diff. F = diffuse Prussian-blue reaction of bone marrow RE cells, Haemosiderin = polymorphous haemosiderin aggregates in bone marrow RE cells, ^{59}Fe-AWBRT = ^{59}Fe-absorption whole body retention test, TIBC = total iron binding capacity UIBC = unsaturated iron binding capacity

in both groups of table II. Significantly lower correlation coefficients were obtained in the comparison of the haemosiderin aggregates, the ^{59}Fe AWBRT TIBC and UIBC, as well as between plasma iron, transferrin saturation and sideroblasts. The red cell indices showed no correlation with the other variables. Correlation coefficients $r > 0.4$ were sufficiently high to reject the null hypothesis at a level of $P < 0.01$ The confidence intervals (table II) show that the differences between most values are statistically significant. Factor analysis applied to the correlation matrices subdivided the variables into the following groups (1) diffuse iron, haemosiderin, ^{59}Fe AWBRT TIBC and UIBC (2) plasma iron, transferrin saturation and sideroblasts (3) red cell indices (r between haematocrit, haemoglobin and erythrocytes + 0.86 to + 0.95)

The scatter diagram of figure 2 reveals the bivariate distribution of cytochemical iron and iron absorption. The arithmetic means of the

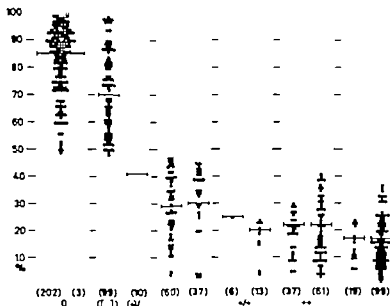


Fig. 2. Intestinal iron absorption (0.56 mg Fe++) in relation to RES-iron of bone marrow. Scatter diagram. Bivariate distribution of types of bone marrow reticuloendothelial iron and iron absorption (%). RES-iron: ○ diffuse iron only, ● diffuse iron with haemosiderin particles (the latter irrespective of their cytochemical grade)

^{59}Fe AWBRT increased significantly (P < 0.01) with the disappearance of the diffuse iron (fig 2, table III B). The same was true in homogenous clinical groups (table III A). Overlapping occurred in the transition zone between normosiderosis and hyposiderosis (fig 2). When diffuse iron was present together with polymorphous haemosiderin particles (fig 2, closed circles, table III B) no significant differences were observed in comparison with the diffuse iron ≥ 1 + alone in normosiderosis (fig 2, open circles).

In different stages of iron deficiency increased iron absorption returned into normal range only after repletion of RE iron stores (table I B-D). In the subjects of table I B diffuse iron 1+ or more was seen after a total dose of 6.0–12.0 g of divalent iron given for 60–120 days (50 mg fasting 1 hour before breakfast and 50 mg at least 2 h after dinner). Intravenous iron therapy (1.0–3.5 g saccharated iron oxide, Ferrophor®) (table I C and D) was characterized by the appearence of numerous uniform sized granules 2+ and more, but

Table III. Iron absorption

Iron absorption	n	AM %	SD % ±	Range %
A. Iron absorption in clinically homogenous groups				
Iron deficiency	64	86.6	12.1	53.4–100.0
Latent iron deficiency	27	85.9	11.7	53.9–100.0
Prelatent iron deficiency	105	74.6	14.8	[illegible]0.0–100.0
Normal females with normosiderosis	38	29.6	11.5	4.0– 50.4
Normal males with normosiderosis	36	25.4	10.7	8.2 44.1
B. Iron absorption in groups with different grades of iron				
1 Diffuse iron without haemosiderin particles				
0	202	84.9	12.6	49.1 100.0
(+)	99	69.5	17.3	10.8– 99.4
+	60	30.8	16.0	1.4– 88.9
+/++	6	25.2	11.6	5.9– 38.7
++	37	21.5	10.2	4.5– 48.6
>++	19	16.9	9.3	3.8– 38.4
	423			
2. Diffuse iron with haemosiderin particles (the latter irrespective of their cytochemical grade)				
0	3	56.0	43.8	12.3–100.0
(+)	10	49.0	26.9	8.2 80.6
+	37	31.3	17.4	0.6– 87.4
+/++	13	19.6	9.5	4.1 40.5
++	61	20.3	11.4	3.2 45.5
>++	99	16.8	9.8	1.4– 49.6
	223			

number of subjects examined, *AM* arithmetic mean of iron absorption values, *SD* standard deviation.

transformation into the diffuse iron varied considerably from 0 to 3+ Relapse of iron deficiency occurred in patients with high ^{59}Fe whole body turnover rates despite of persisting 'residual bodies 1+ to 4+ and diffuse iron O (table IC, n = 15 in part other patients than n = 16)

In table IV the 780 subjects examined have been subdivided according to the clinical interpretation of iron absorption in relation to iron stores. Findings classified as abnormal have been confirmed as far as possible by repeated tests. Normal iron absorption in prelatent iron deficiency (table IV B1) increased in single cases with progression

Table IV Iron absorption

		Number of subjects	% of subjects
A. Iron absorption in relation to RES iron			
1. Increased absorption > 50% associated with hyposiderosis		304	39.0
2. Normal absorption < 50% associated with normosiderosis (or hypersiderosis)		422	54.0
		776	93.0
B. Iron absorption unrelated to RES iron			
Deviations of minor significance			
1. Normal absorption despite of prelatent iron deficiency	No./total 9/114	9	1.2
2. Normal absorption despite of neg. RES iron in advanced lymphocytic leukemia	6/ 47	16	2.1
myelocytic leukemia	10/ 17		
3. Unexplained increase of absorption despite of normosiderosis		7	0.9
Deviations of major importance			
4. Normal absorption despite of iron deficiency anaemia (hereditary malabsorption functional disturbances?)	(2/ 66)	2	0.[illegible]
5. Increased absorption despite of normosiderosis or hypersiderosis in sideroachrestic anaemia	7/ 14	13	1.7
megaloblastic anaemia	3/ 10		
haemolytic anaemia	2/ 11		
renal anaemia	1		
haemochromatosis	4/ 7	7	0.9
chronic hepatitis	3		
		54	7.0
Sum A+B		780	100.0

of iron deficiency. Unexplained high iron retention despite of normosiderosis (table IV B3) returned spontaneously into normal range in 4 cases and persisted in 3 subjects. Far advanced chronic leukemia (table IV B2) was characterized by heavy bone marrow infiltration.

Normal iron absorption was associated with severe iron deficiency anaemia in two sisters showing poor response to oral iron, but ex-

cellent effect of parenteral iron (table IV B4) The ^{59}Fe whole body turnover rate was normal in 1 case.

In sideroachrestic anaemia increase of iron absorption into sideropenic range was found together with latent and manifest iron overload, in haemolytic and megaloblastic anaemia together with latent and prelatent iron overload (table IV B5) The group of haemochromatosis with increased ^{59}Fe retention consisted of 1 idiopathic case and 3 alcoholics.

Ten patients with osteomyelosclerosis without reliable estimation of bone marrow iron and without symptoms of iron deficiency showed normal absorption. Three individuals with splenectomy and hyposiderosis were included into group A 1 (table IV) 15 corresponding subjects with normosiderosis or hypersiderosis into group A 2.

Discussion

The data presented concern clinical problems studied already since many years [5] with a growing refinement of methods and an increasing number of individuals. Positive correlations between plasma iron, transferrin saturation and sideroblasts (table II) are in accordance with the work of others [32] In comparison of iron stores and intestinal iron absorption less variability [20] has been achieved after better recognition of the following facts probably not fully considered in the past

1 Special care must be given in every case to the instruction and co-operation of the subjects to be examined because iron absorption decreases by the factor 2–3 or more when the individuals are not completely fasting (table I A) during a critical period of about 10 h before and 2 h after the administration of the test dose. Dietary and secretory factors seem to render the divalent iron ascorbate less easily absorbable [8] Unregulated intraluminal inhibition of iron absorption is to be suspected when low or normal values are found in subjects with hyposiderosis, but corresponding observations can be made in every group. These irregular effects disappear usually in the course of repeated examinations after the confrontation of the subjects with the probable cause of abnormal results. Unexpected isolated mal absorption of iron as found in two sisters with long standing iron deficiency anaemia and poor response to oral iron for several years

(table IV) should be established by normal or low values in more than one test. In prelatent iron deficiency normal ^{59}Fe retention (table IV) can increase with progression of iron deficiency (functional disturbance?)

2. Heavy blood losses during the first 14 to 21 days after iron loading and ^{59}Fe whole body turnover rates of more than 1–2% daily can lead to erroneously too low results in measurement of iron absorption.

3 The diffuse cytoplasmatic Prussian blue reaction of bone marrow reticulo-endothelial cells is more closely correlated with the ^{59}Fe retention test than the polymorphous haemosiderin particles, mobilized slowly (table II, fig 2) These findings are of clinical significance because important work on the evaluation of iron stores has been done using unstained preparations which reveal the haemosiderin aggregates only [3 28, 30] Except by BEUTLER *et al.* [4] it has been not described [10 23 27 29 32] whether or to what extent the diffuse iron is included into cytochemical grading of iron.

4 To obtain groups with distinct diagnostic ranges of iron absorption it is necessary to classify exhausted iron stores with normal plasma iron and red cell indices as the first, 'prelatent' stage of iron deficiency which is a frequently observed symptomatic, co-morbid and co-normal phenomenon showing increased iron retention. Without this manipulation adequate organization of data is much more difficult.

For practical purposes cytochemical grading of non haeme iron in bone marrow reticuloendothelial cells as described can be regarded as the most appropriate single reference system for the clinical interpretation of iron absorption levels (table IV) The disappearence of a faint blue diffuse staining of bone marrow fragments and isolated macrophages in more than 25 to 50% of comparable microscopic fields coincided with an increase of iron absorption over 50% (table I, III fig 2) This criterium seems to be a suitable dividing line between normosiderosis and hyposiderosis (fig 2) Consideration of the total or unsaturated iron binding capacity gives less clear cut results in the diagnosis of early iron deficiency

In 93% of the subjects examined the findings indicate that iron absorption is regulated in relation to the reticuloendothelial iron deposits which are present in the bone marrow liver spleen and the lamina propria of the duodenal and jejunal mucosa. This rule concerns healthy subjects and the majority of diseases independent of age and sex. The iron storing capacity of the subepithelial macrophages in the

upper small intestinum after oral or parenteral iron administration in animal experiments has been studied histochemically 100–40 years ago by numerous authors in relation to questions of iron absorption and iron excretion [2, 7 18, 25] Recently interest in these cells has been revived [1 2 7 9 25, 34] The subepithelial macrophages are thought to act as transitory iron stores in iron absorption [2] or to contribute to iron excretion in iron overload when they transverse the columnar epithelium [1 2, 9] Intravenous application of 1 0–3.5 g saccharated iron oxide normalizes the increased iron absorption in all stages of iron deficiency (table I) Diffuse iron similar to that of the bone marrow has been seen in squash preparations and sections of biopsy material obtained during surgery from the jejunum only in the tips of the villi [unpublished] Uniform sized iron granules smaller than the corresponding particles in the marrow were observed in the adjacent endothelial cells. Furthermore the subepithelial RES is suspected to phagocytize red cells.

The available data indicate that except in far advanced chronic leukemia (table IV) and severe osteomyelosclerosis the diffuse reticuloendothelial iron of the marrow reflects rather reliably the intestinal subepithelial iron pool connected with mucosal inhibition of iron absorption when the macrophages are located in the tips of the villi, but probably less effective when these cells are dispersed irregularly within the lamina propria [1] It is therefore not surprising that splenectomy removing a considerable part of the iron storing RES does not influence iron absorption. A proportionate negative feed back is suggested to occur in the tips of villi between the subendothelial RES cells iron laden from different sources (iron absorbed from the lumen, aged red cells and colloidal iron taken up from the blood vessels) and the absorbing epithelium iron laden in part in relation to the plasma iron [9] This working hypothesis is flexible enough to explain the findings reported and to induce further studies.

A normal or elevated plasma iron up to 270 µg% does not substitute depleted iron stores in regulation of iron absorption as shown in prelatent iron deficiency (table III) (feed back deficiency) On the other hand in malignant diseases and infections normal or increased iron stores are sufficient to compensate extreme hyposideraemia which did not increase iron absorption in these cases [14] (feed back enhanced) In acute bleeding anaemia only or in pernicious anaemia after treatment with vitamin B_{12} during reticulocytosis low plasma

iron and anaemia seem to act additively to elevate iron absorption before the iron stores are exhausted [unpublished]. The increase of iron absorption into sideropenic range observed in a varying percentage of patients with sideroachrestic anaemia, pernicious anaemia, and haemolytic anaemia (table IV) despite of normosiderosis or different degrees of hypersiderosis, but not in hypoplastic anaemia [13] can be attributed to the combined effect of severe anaemia, high plasma iron turnover and unequal distribution of iron within the RES (inhibition of feed back?). In such cases it remains to be explored whether histochemistry and cytochemistry of non haeme iron in the intestinal mucosa can reveal morphological differences to haemochromatosis [1, 7, 34]. In the latter condition a hereditary or acquired feed back insufficiency with increased ^{59}Fe retention can perhaps be compensated by further increase of iron overload leading to normal absorption again [5]. Studies on the correlations between the diagnostic criteria of iron metabolism including the histochemistry and cytochemistry on iron in the columnar epithelium, the macrophages and the endothelial cells of the mucosa of the upper small intestine, will probably give more detailed insight in the regulation of iron absorption, but it should be considered that the Prussian blue reaction reveals only a part of the non haeme iron.

Summary

The diagnostic criteria of iron metabolism have been compared in 780 subjects (normal controls, patients with blood diseases, liver diseases, malignant diseases and infections). A high negative correlation has been computed between the diffuse Prussian blue reaction of bone marrow reticuloendothelial cells and the intestinal iron absorption. In 93% of the individuals examined the results indicate that iron absorption is regulated in relation to the reticuloendothelial iron stores in normal controls and the vast majority of diseases.

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Authors' addresses Dr. K. Hausmann and Dr R. Kuse, Hämatologische Abteilung, Allg. Krankenhaus St. Georg, 2 Hamburg 1; O. W Schwarzenberg, Rechenzentrum der Universität, Dr H. Bartels and Prof. H. C. Heinrich, Abteilung für Med. Biochemie, Physiol.-chem. Institut der Universitätsklinik Eppendorf, 2 Hamburg 20 (Germany).

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Medizinische Universitätsklinik (Direktor: Prof. H. E. Bock) Tübingen

Zytologische Untersuchungen zur Diagnostik und Aetiologie der Erythroleukämie

D. Müller[1]

Über das Syndrom Erythroleukämie liegen zahlreiche Veröffentlichungen vor. Publizierte Krankheitsabläufe und angewandte Nomenklatur weichen jedoch z.T. erheblich voneinander ab [1–6]. Nach Heilmeyer [3] handelt es sich bei Erythroleukämien um neoplastische Bluterkrankungen, bei denen die Erythropoese isoliert wie bei der akuten Erythrämie di Guglielmo und der chronischen Erythroblastose Heilmeyer-Schöner [4] oder zusammen mit anderen Zellsystemen befallen ist. Dameshek und Gunz [2] nehmen an, dass alle Formen nur verschiedene Stadien einer Erkrankung darstellen, wobei der Beginn als Erythrämie, spätere Phasen als finaler Myeloblastenschub [5] imponieren können. Sie wenden sich damit indirekt gegen die Existenz einer isolierten Erkrankung der Erythropoese und setzen eine gleichzeitige leukämische Umwandlung weisser und roter Blutzellen voraus.

Wann ist man aber berechtigt, bei vermehrtem Auftreten roter Vorstufen in Blut und Knochenmark im Ablauf einer akuten Leukämie das Vorliegen einer echten Erythroleukämie anzunehmen und eine Begleiterythroblastose gleich welcher Genese auszuschliessen? In den letzten Jahren sind an leukämischen Zellen neue zytologische Befunde erhoben worden, die an den roten Vorstufen bei Kranken mit akuter Leukämie für die Diskussion um das Krankheitsbild Erythroleukämie zu überprüfen sind. 1. Gegenüber der normalen Granulopoese finden sich bei leukämischen Zellen eingreifende Veränderungen in der Art

[1] Mit Unterstützung der Deutschen Forschungsgemeinschaft

und Weise der Zellvermehrung. Dies konnte durch zahlreiche Untersuchungen vorwiegend nach ³H-Thymidin-Inkubation gesichert werden [7–11]. 2. Bestehen in einer erheblichen Anzahl akuter Leukämien Chromosomenaberrationen, die die pathologische Entwicklung dieser Zellen unterstreicht. Bei chronisch myeloischen Leukämien tritt fast regelmäßig das Philadelphia-Chromosom [12] auf, das deshalb als pathognomonisch für diese Erkrankung angesehen werden kann, wenngleich auch Ausnahmen beschrieben sind [13, 14]. 3. Schließlich gestatten moderne zytochemische Untersuchungen eine immer eingehendere und detailliertere Unterscheidung der Blutzellen, wobei allerdings Untersuchungen an verschiedenen Formen akuter Leukämien [15–17] gezeigt haben, daß die leukämisch entarteten Zellen in ihrer zytochemischen Beschaffenheit auffallende Ähnlichkeit mit dem Ausgangs- bzw. Muttergewebe aufweisen.

Methodik

1. Qualitative zytochemische Untersuchungen: alkalische Phosphatase, Azokupplungsreaktion nach KAPLOW [18], Methode nach MERKER und HEILMEYER [19]; saure Phosphatase nach LÖFFLER und BERGHOFF [20]; alpha-Naphthylacetatesterase, Methode nach LÖFFLER [21]; PAS-Nachweis [22]; Sideroblasten-Nachweis [23] mit der Berliner-Blau-Reaktion.

2. Semiquantitativer Nachweis der Kern-DNS mit Hilfe der Feulgen-Reaktion [24]: Formol-Fixation, 12 min (9–15 min) Hydrolyse in 1 N HCl bei exakt 60° C (Thermostat), 60 min Inkubation in Schiffschem Reagens, Waschen in Kaliummetabisulfit, aufsteigende Alkoholreihe, Eindecken in Caedax (n/D 20° = 1,55) [25]. Zytophotometrie mit dem Mikrospektrophotometer UMSP I von Zeiss [25, 26].

Krankengut

Von den in den letzten Jahren beobachteten Patienten mit akuter Leukämie wurden bei 58 nicht ausgewählten Fällen zytochemische Untersuchungen durchgeführt. Vergleichende Untersuchungen an Erythroblasten wurden bei 24 Patienten durchgeführt, bei denen der Anteil roter Vorstufen im Knochenmark im Krankheitsablauf z.T. über 10% lag. Bei 4 dieser Kranken wurden gleichzeitig quantitative DNS-Untersuchungen nach Feulgen-photometrie in der oben angegebenen Methodik ausgeführt. Zur Auswertung der Meßergebnisse siehe bei [26]. Für Vergleichsuntersuchungen wurden Kranke mit normaler Erythro- und Granulopoese sowie mit sideroachrestischen Anämien [27] herangezogen.

Befunde

Die zytologische Differenzierung der akuten Leukämien erfolgte nach den früher angegebenen Kriterien [27]. Die Ergebnisse qualitativer zytochemischer Untersuchungs-

Tabelle I Qualitative zytochemische Untersuchungsergebnisse an der Erythropoese bei akuten Leukämien

Leukämieform zytochemisch differenziert	Fallzahl	Reti ‰	Normoblasten in der Peripherie, %	Knochenmarkbefund			
				Anteil roter Vorstufen auf 100 weiße Zellen	Sideroblasten % (normal: 20–40)	PAS + rote Vorstufen % (normal < 2)	stark (+ + + +) α-Naphthylacetatesterase-positive rote Vorstufen (Fallzahl)
Undiff.	3	10,6	1,1	19,3	30,3	6,9	0
Myeloisch	11	18,8	1,8	10,5	34,1	10,6	2
Monozytoid	2	30,5	< 1	18,0	37 0	4,5	0
Lymphoid	8	13,8	< 1	5,7	37,0	5,7	0

methoden an der Erythropoese bei akuten Leukämien gehen aus *Tabelle I* hervor. Eine stark positive Esterasereaktion (+ + + +) findet sich im Zytoplasma roter Vorstufen bei 2 Kranken mit myeloischen Paraleukoblastenleukämien. Ebenfalls findet sich der höchste Anteil PAS-positiver Erythroblasten bei myeloischen Paraleukoblastenleukämien. Der Anteil der Sideroblasten liegt im Durchschnitt aller Fälle im oberen Normbereich. Eine sichere Beziehung zwischen diesen zytochemischen Befunden und dem Anteil der roten Vorstufen im Knochenmark sowie dem Prozentsatz der in der Peripherie nachgewiesenen Normoblasten und Retikulozyten besteht nicht (Tab. I).

Bei quantitativen Feulgen-photometrischen DNS-Bestimmungen in der Einzelzelle weist die Mehrzahl normaler Myelozyten, Promyelozyten und Myeloblasten bei der von uns angewandten Methodik der Zytophotometrie bei 28 Kontrollen einen Mittelwert von 35,0 Arbeitseinheiten (AE) auf (35,0 ± 2,8 AE, Toleranzbereich 95% für 95% der Verteilung [27]). Der Rest der Zellen zeigt eine höhere Feulgenanfärbung. In nicht mehr teilungsfähigen segmentkernigen Granulozyten liegt die Feulgenanfärbung niedriger (31 1 ± 0,8 AE im Durchschnitt von *10 Fällen* [27]). Dasselbe gilt für nicht mehr teilungsfähige Normoblasten [27], weshalb die Mehrzahl aller kernhaltigen roten Vorstufen im Durchschnitt eine geringere Feulgenanfärbung (32,0 AE [27]) als Myelozyten, Promyelozyten und Myeloblasten aufweist. Das Maximum der Feulgenanfärbung wird in segmentkernigen Granulozyten, Granulozytenvorstufen und in roten Vorstufen nach einer 12minütigen Hydrolysedauer erreicht. Eine Hydrolysezeit von 9 wie von 15 min ergibt eine niedrigere Feulgenanfärbung.

Bei 4 Kranken mit akuter Leukämie ist die stärkste Anfärbung der Paraleukoblasten einmal über den Toleranzbereich normaler Granulozytenvorstufen erhöht, zweimal im oberen Normbereich und einmal im unteren Normbereich gelegen (Tab. II, Abb. 1). Die Feulgenanfärbung der Erythroblasten zeigt in allen 4 Fällen gleichartige Veränderungen. Die stärkste Feulgenanfärbung ist in 2 Fällen erst nach 15 min Salzsäure-Hydrolyse erreicht. Leukämiezellen und Erythroblasten verhalten sich identisch, während die am gleichen Präparat untersuchten segmentkernigen Granulozyten wie bei den normalen Kontrollen ihre stärkste Feulgenanfärbung nach 12minütiger Hydrolysedauer aufweisen.

Der Zellanteil mit erhöhter Feulgenanfärbung als Zeichen der prämitotischen DNS-Synthese war in den selben Fällen (von denen 3 noch unbehandelt waren) bei den Paraleukoblasten und auch bei den roten Vorstufen gegenüber normalen Kontrollen vermindert (Tab. II, Abb. 1).

Tabelle II. Qualitative und quantitative zytochemische Befunde bei 4 Kranken mit akuter Leukämie

	Myeloische Paraleuko-blastenleukämie		Monoz. Para-leukobl. L.	Undiff. Pa-raleukobl. L.
	Ro. ♂ 42 J	Ral. ♀ 60 J	Kr ♀ 52 J	Ve. ♀ 32 J
Peripheres Blut				
Reti, ‰	6	20	5	14
Normoblasten, %	3	12	0	2,5
Knochenmark				
Anteil roter Vorstufen auf 100 weisse Zellen	7	45,0	7	31
Sideroblasten, %	50	8	12	46
PAS + rote Vorstufen, %	12	34	8	3
Stark (+ + + +) α-Naphthylacetatesterase positive rote Vorstufen	Ø	+	Ø	Ø
Mittlerer DNS-Gehalt in Feulgen-AE in				
G_1-Paraleukoblasten	39,4	32,9	37 1	37,2
G_1-Erythroblasten	37 7	32,3	36,7	34 4
Zur stärksten Feulgenanfärbung erforderliche Hydrolysezeit, min				
Paraleukoblasten	12	9	15	15
Erythroblasten	12	12	15	15
Vorstufen mit erhöhter Feulgenanfärbung als Zeichen der DNS-Synthese, %				
Paraleukoblasten	< 1	3	1,5	< 1
Erythroblasten	7	14	2	17

Normale Myeloblasten, Promyelozyten, Myeloblasten 33,0 ± 2,8 AE [27]; rote Vorstufen 32,0 AE [27].

Normale Myeloblasten, Promyelozyten, Myelozyten 37,2% (Mittelwert von 12 Fällen [27]), normale rote Vorstufen 33,5% (Mittelwert von 7 Fällen [27]).

Diskussion

Bei Kranken mit akuter Leukämie wurden qualitativ und quantitativ zytochemische Untersuchungen unter der Fragestellung durchgeführt, ob mit den angewandten Methoden eine leukämische Umwandlung in der Erythropoese erkannt werden kann. Die Suche nach

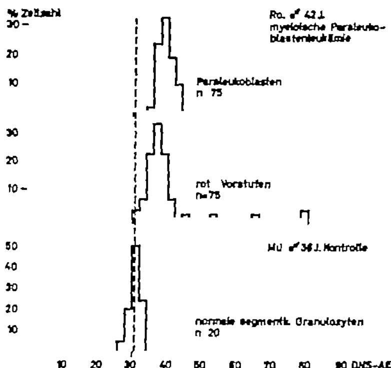


Abb. 1 DNS-Gehalt in weissen und roten Vorstufen des Knochenmarks. Ordinate = Zellzahl, Abszisse = relativer DNS-Gehalt in Feulgen-AE. Oben erhöhter DNS-Gehalt in weissen und roten Vorstufen bei myel. Paraleukoblastenleukämie. Sehr wenig Zellen mit höherem DNS-Gehalt als Zeichen der interphasischen DNS-Reduplikation. Unten normale segmentkernige Granulozyten. Feulgenfärbung nach 12 min Salzsäurehydrolyse.

objektiven Kriterien zur Beantwortung dieser Frage gründet sich darauf, dass rein morphologische Kriterien keine ausreichende Antwort bieten können. Die rein zahlenmässige Vermehrung roter Vorstufen in der Blutperipherie mag in einzelnen Fällen beweisend für das Vorliegen einer Erythroleukämie sein. Wie verhält es sich aber wenn durch eine zytostatische Therapie sowohl der Anteil leukämischer weisser Blutzellen wie der Anteil roter Vorstufen in der Blutperipherie vermindert ist, oder wenn es sich, wie bei einem Teil der akuten Leukämien, von Beginn an um eine leukopenische Verlaufsform handelt? Wie reagieren in diesem Fall die roten Vorstufen? Auch die qualitativen Kriterien in der Morphologie der roten Vorstufen nach Pappenheimfärbung sind zum Teil subjektiv und kaum geeignet, als einziger Parameter für die Diagnostik einer Erythroleukämie zu dienen.

Nachdem verschiedene zytologische Untersuchungsmethoden in der Hämatologie Eingang fanden, wurden diese Techniken auch für die Differenzierung von Erythroleukämien herangezogen. HAYHOE *et al.* [28] die 140 Kranke mit akuter Leukämie untersuchten, fanden in Fällen mit reichlich roten Vorstufen in der Blutperipherie gehäuft PAS-positive Substanzen im Zytoplasma der Erythroblasten. Dieser Befund wurde von VERLOOP [29] bestätigt und eine gleichzeitige Vermehrung der Sideroblastenzahl beschrieben. Schließlich fand LEDER [31] dass sich in den Erythroblasten bei Erythroleukämien eine besonders hohe Aktivität der alpha Naphthylacetatesterase im Zytoplasma nachweisen lässt.

Ähnliche Befunde wurden von uns an den Erythroblasten bei allen Kranken mit akuten Leukämien erhoben Sie können deshalb nicht spezifisch für das Vorliegen «leukämischer» Erythroblasten sein. So fanden KLEIN *et al* [30] fast stets PAS-positive Substanzen, also eine Anhäufung von Glykogen, Gluko- und Mukoproteinen in nekrobiotischen Erythroblasten. Da solche Zellen nicht mehr weiter ausreifen, ist dieser Befund ein Zeichen einer ineffektiven Erythropoese. Dem entspricht der Nachweis PAS-positiver Substanzen im Zytoplasma roter Vorstufen bei sideroachrestischen Anämien [29] bei denen das Vorliegen einer ineffektiven Erythropoese durch ferrokinetische Untersuchungen bekannt ist. Dieser Befund kann damit keineswegs spezifisch für eine Erythroleukämie sein.

Auch die Vermehrung der Sideroblasten weist lediglich auf eine Störung der Eisenutilisation bei der Hämoglobinsynthese hin. Bestehen bleibt die von LEDER [31] angegebene erhöhte alpha Naphthylacetatesteraseaktivität in «leukämischen» Erythroblasten, die in der Literatur bisher noch wenig diskutiert wurde.

Bei der Abgrenzung einer Erythroleukämie mit zytochemischen Methoden betonen deshalb auch HAYHOE *et al.* [28] dass die Übergänge zwischen den Fällen mit einem hohen und einem niedrigen Anteil PAS-positiver Erythroblasten fliessend sind. Es handelt sich um ein durch nichts begründetes Postulat, wenn die Autoren bei einem Anteil von 10 und mehr Prozent PAS-positiver Erythroblasten das Vorliegen einer Erythroleukämie annehmen. So nimmt es nicht wunder dass die eigenen qualitativen zytochemischen Untersuchungsergebnisse eine eindeutige Differenzierung einer Erythroleukämie nicht ermöglichen. Den höchsten Anteil PAS-positiver roter Vorstufen beobachteten wir unter den myeloischen Paraleukoblasten-

leukämien aber auch im Durchschnitt der anders differenzierten Leukämieformen fanden sich noch mehr als 5% PAS-positive rote Vorstufen. Die Sideroblasten waren zum Teil deutlich vermehrt, wiesen aber auch Beziehungen zur wechselnden Höhe des Eisenspiegels im Serum auf. Sehr stark Esterase positive rote Vorstufen fanden sich nur in zwei Fällen.

Eine schwere Störung in der Art der Zellneubildung wurde durch autoradiographische Untersuchungen mit ³H Thymidin von zahlreichen Autoren [7–11] bei leukämischen Zellen nachgewiesen. Im Gegensatz zu den normalen Blutzellen stehen viel weniger leukämische Zellen in der Phase der prämitotischen DNS-Synthese. Dadurch ist zwar die Zellneubildung verlangsamt, durch eine gleichzeitige Störung der Zellausreifung bleiben jedoch mehr Zellen weiter teilungsfähig, weshalb es trotzdem zu einer schnellen Vermehrung der Gesamtzellzahl kommen kann. Ein herabgesetzter Anteil roter Vorstufen in DNS-Synthese wurde nach ³H Thymidin Autoradiographie von Gavosto *et al.* [41] auch bei 2 Kranken mit einem di Guglielmo-Syndrom beobachtet. Unsere Untersuchungen an der Erythropoese bei 4 Kranken mit einer akuten Leukämie zeigten einen herabgesetzten Anteil roter Vorstufen wie Paraleukoblasten in DNS-Synthese (Tab. II) Nur einer dieser Fälle liess klinisch an das Vorliegen einer Erythroleukämie denken. Da drei der Kranken noch unbehandelt waren, kann der Befund nicht durch eine zytostatische Therapie verursacht sein. Die einzige Erklärung für diese Beobachtung scheint die Annahme einer gleichartig auftretenden Störung der Zellregeneration in verschiedenen Blutzellsystemen während der Leukämogenese zu sein.

Strukturelle und numerische Chromosomenaberrationen werden bei leukämischen Erkrankungen häufig beobachtet [32] Sie treten auch bei Erythroleukämien auf. Da aber eine sichere Zelldifferenzierung an der gestreuten Metaphase in der Zellkultur wie am Knochenmark Direktpräparat nicht möglich ist, gelingt eine eindeutige Zuordnung einer ausgezählten Metaphase zur Granulo- oder Erythropoese im Einzelfall nicht. Deshalb kommt bei der engen Korrelation zwischen DNS-Gehalt und Chromosomenzahl [35] den eigenen getrennten quantitativen feulgenphotometrischen Kern-DNS-Messungen in den roten und weissen Vorstufen bei akuten Leukämien eine besondere Bedeutung zu. Bei allen 4 Kranken mit einer akuten Leukämie fanden wir enge Beziehungen zwischen dem DNS-Gehalt der leukämischen Blasten und dem der roten Vorstufen und zwar so-

wohl bei zwei myeloischen Paraleukoblastenleukämien als auch bei einer monozytoiden Paraleukoblastenleukämie und einer nicht näher differenzierbaren akuten Leukämie. Ausserdem zeigten sich an der Erythropoese gleichartige qualitative Veränderungen des Kernchromatins wie an den leukämischen Blasten. Bei zwei Kranken mit einer myeloischen Paraleukoblastenleukämie fand sich in leukämischen Blasten wie in der Erythropoese die stärkste Feulgenanfärbung nach 12 min Salzsäurehydrolyse. Bei einem Kranken mit einer monozytoiden Paraleukoblastenleukämie und einem anderen Kranken mit einer undifferenzierten Paraleukoblastenleukämie war die stärkste Feulgenanfärbung der leukämischen Blasten wie der roten Vorstufen erst nach 15 min erreicht. Dabei sprach gegen einen technischen Fehler das unveränderte Verhalten der verbliebenen normalen segmentkernigen Granulozyten (Anfärbung nach 12 min stärker als nach 15 min Hydrolyse). Ob die Empfindlichkeitsänderung der DNS gegenüber Salzsäurehydrolyse durch eine veränderte molekulare DNS-Histon-Bildung verursacht ist, lässt sich nicht sagen. Von Bedeutung ist jedoch das gleichartige Verhalten roter Vorstufen und pathologischer weisser Blutzellen.

Die quantitativen feulgenphotometrischen DNS-Befunde in der Erythropoese bei akuten Leukämien lassen uns den Schluss ziehen, dass im Einzelfall eine sichere Unterscheidung zwischen einer «leukämisch entarteten» Erythropoese und einer normalen Erythropoese bei Leukämie nicht möglich ist [u. a. 43, 49]. Die roten Vorstufen zeigen viel mehr bei den von uns untersuchten Fällen Veränderungen, die den pathologischen Befunden an den leukämischen weissen Blutzellen ähneln. Wir sehen darin einen Hinweis dafür, dass die leukämische Zellumwandlung nicht *ein* Zellsystem, sondern *mehrere* Blutzellsysteme gemeinsam erfasst und nur in einem Zellsystem stärker in Erscheinung tritt. Für diese Annahme sprechen die folgenden Überlegungen: Ein Philadelphia-Chromosom als scheinbar leukämiespezifische Chromosomenaberration wurde schon vor Jahren nicht nur in den weissen Blutzellen, sondern auch in roten Vorstufen [34] ohne einen Anhalt für eine «leukämische» Erythropoese gefunden. Die hier beschriebenen qualitativen und quantitativen DNS-Befunde sind in Paraleukoblasten wie in roten Vorstufen bei myeloischer und monozytoider Paraleukoblastenleukämie gleichartig. Eine Störung der Zellneubildung findet sich in den weissen und roten Blutzellen. Ähnliche Veränderungen der Zellregeneration und des Kernchromatins mit Chromo-

somenabberationen [36 37] werden in der Erythro- und Granulopoese bei Panzytopenien beobachtet [27 42] die manche Autoren als präleukämische Stadien ansehen [37–40]

Zusammenfassung

Bei 24 Kranken mit akuter Leukämie wurden zytochemische Untersuchungen an der Erythropoese durchgeführt. Es fand sich eine Zunahme PAS-positiver Erythroblasten, Sideroblasten und stark Esterase-positiver Erythroblasten. Feulgen-photometrische DNS-Untersuchungen zeigten in der Erythropoese ähnliche Veränderungen des Kernchromatins wie in den leukämischen Blasten. Der Anteil roter Vorstufen in prämitotischer DNS-Synthese war ähnlich wie in den leukämischen Blasten herabgesetzt.

Die fliessenden Übergänge zwischen normalen und pathologischen Befunden in der Erythropoese lassen eine Differenzierung zwischen Leukämie und Erythroleukämie mit den angewandten Methoden nicht zu. Die an roten Blutzellen wie an Paraleukoblasten beobachteten ähnlichen Veränderungen stützen die Vorstellung, dass es während der Leukämieentstehung zu einer gleichartigen Störung an granulopoetischen, monozytären und erythropoetischen Zellen kommt.

Summary

Cytochemical studies of erythropoiesis were carried out on 24 patients with acute leukaemia. Increased numbers of PAS-positive erythroblasts, sideroblasts and strongly esterase-positive erythroblasts were seen. Determination of DNA by the Feulgen photometric method showed similar changes in erythropoiesis as in leukaemic blast cells. The proportion of red precursors in premitotic DNA synthesis was reduced as in leukaemic blasts.

The continuous transition from normal to pathological findings in erythropoiesis make it impossible to distinguish between leukaemia and erythroleukaemia by the methods used. The similar changes observed in red cells and paraleukoblasts lend support to the view that there is simultaneous disturbance of monocytes, granulopoietic, and erythropoietic cells in the development of leukaemia.

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Adresse des Autors: Dr. D. Müller, Medizinische Universitätsklinik, 74 Tübingen (Deutschland).

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Institut de Biochimie, Université de Lausanne, Lausanne

Etude de la fixation non spécifique des immunoglobulines G sur les érythrocytes

II. Essais d'inhibition par des immunoglobulines G natives ou modifiées et par d'autres protéines

D. Des Gouttes et H. Isliker

Dans un premier travail [2], la fixation non spécifique des immunoglobulines G (IgG) sur les érythrocytes a été étudiée en fonction de la variation de différents paramètres: la quantité d'IgG, le nombre d'érythrocytes, le pH, la force ionique, la température, la durée d'incubation et le lavage des érythrocytes après l'incubation. Des conditions expérimentales permettant d'obtenir des résultats reproductibles ont été déterminées. La fixation des IgG sur le verre a également été considérée. Le présent travail étudie l'inhibition de la fixation de l'IgG marquée au ^{125}I par l'IgG native ou modifiée ou par d'autres protéines non marquées capables de prendre la place de l'IgG marquée au site de fixation cellulaire. Par analogie aux expériences d'inhibition de la fixation spécifique [5–9], cette étude a pour but de préciser s'il existe sur la molécule d'IgG une structure bien définie responsable de la fixation cellulaire non spécifique.

Matériel et méthodes

La préparation des érythrocytes, la purification et la iodation de l'IgG ont été décrites dans le précédent travail [2].

La digestion à la pepsine de l'IgG a été faite selon la méthode de Utsumi et Karush [14] en incubant l'IgG avec la pepsine (Worthington, Freehold, N.J., USA) pendant 20 h à 37°C dans un tampon acétate 0,1 M pH 4,5.

La préparation des fragments d'IgG a été faite selon la méthode de Porter [10] en utilisant de la papaïne deux fois cristallisée (Worthington, Freehold, N.J., USA) dans un tampon acétate 0,1 M, pH 7,0 en présence de cystéine 0,01 M et d'EDTA 0,002 M. Après séparation des produits de digestion sur CM-cellulose, *le fragment Fab* a été purifié sur Sephadex G-100 ou G-150 en utilisant comme éluant du bicarbonate d'ammonium 0,05 M. *Le fragment Fc* a été purifié selon la méthode de Prahl [11]. Le coefficient de sédimentation des

fragments F b et Fc été estimé à 3,4 S. La pureté des différentes fractions utilisées été démontrée par la technique d'immuno-électrophorèse décrite par Grabar [9] modifiée par Scheidegger [12] et la technique de double diffusion décrite par Ouchterlony [8].

Les antisérums utilisés étaient les suivants sérum de cheval anti-lapin sérum de mouton anti-lapin[illegible] sérum de mouton anti-Fab de lapin[illegible] sérum de mouton anti-Fc de lapin[illegible] Le traitement de l'IgG à pH 2,9 été fait par dialyse pendant 20 h contre un tampon acétate 0,3 M.

Les expériences d'inhibition de la fixation non spécifique de l'IgG-^{131}I sur les érythrocytes ont été faites en mélangeant à l'IgG marquée des protéines non marquées; ce mélange a été incubé avec des érythrocytes, et la radioactivité des culots d'érythrocytes été mesurée. Les données numériques sont exprimées en moyenne ± l'erreur moyenne de la moyenne ($\bar{}$ ± SE) La signification des différences entre les moyennes été calculée en utilisant le test t selon les méthodes statistiques habituelles [15]. Des culots-contrôles incubés avec de l'IgG marquée seule représentent 100% de fixation. La différence, exprimée en pourcent, de la radioactivité entre les culots-contrôles et les culots-test indique l'inhibition de la fixation non spécifique de l'IgG-^{131}I. Afin de pouvoir affirmer que le système d'inhibition par les protéines non marquées est valable, il a été démontré que le processus de iodation ne modifie pas l'affinité de l'IgG pour les cellules. En effet, la fixation de l'IgG-^{131}I aux érythrocytes n est pas influencée différemment par l'adjonction d'IgG non marquée ou d'IgG marquée à l'iode non radioactif. Vu l'absence d'une quantité définie d'IgG saturant la membrane érythrocytaire, les expériences d'inhibition ont été faites en ajoutant à une quantité constante d'IgG-^{131}I des quantités croissantes de protéines non marquées.

Résultats

Inhibition par l'IgG ou la sérumalbumine humaine (HSA) non marquées La fixation de l IgG-^{131}I de lapin aux érythrocytes est mesurée en fonction de l adjonction de quantités croissantes d IgG de lapin ou d'HSA non marquées. Les résultats représentés sur la figure 1 montrent que l effet inhibiteur de ces protéines non marquées est faible, mais il est plus marqué pour l HSA que pour l'IgG Le pourcentage d inhibition produit par l HSA reste presque constant, malgré l adjonction croissante d HSA. Une expérience préalable montre par ailleurs que l HSA ^{131}I ne se fixe pas plus que l IgG-^{131}I sur les érythrocytes.

Inhibition par l'IgG traitée à pH 2,9 Le traitement de l IgG à pH 2,9 produit des modifications de la structure de l IgG Dans ces expériences, la fixation de l IgG ^{131}I sur le verre est incluse dans les valeurs obtenues pour le culot. On étudie l effet inhibiteur de l IgG traitée à pH 2 9 et réajustée à pH 7 4 sur la fixation de l IgG-^{131}I en quantité égale ou 5 fois plus grande. L effet inhibiteur de l IgG exposée à pH 7 4 et celui

Fourni par le Prof. P Grabar, Institut Pasteur Paris (France)
Fourni par le Dr S. Barandun, Tiefenauspital, Berne (Suisse).
Fourni par le Dr T W[illegible], WHO Reference Centre, Lausanne (Suisse).

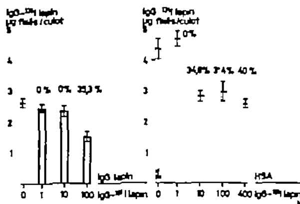


Fig 1 Inhibition de la fixation de l'IgG-^{125}I aux érythrocytes par l'adjonction de quantités croissantes d'IgG ou d'HSA non marquées. ( ) Inhibition avec l'IgG non marquée. Des quantités croissantes d'IgG non marquées ont été ajoutées à 100 µg d'IgG-^{125}I; ce mélange est ensuite incubé vec 8,1 10^{8} érythrocytes. La quantité d'IgG-^{125}I fixée aux érythrocytes est exprimée en µg par culot pour différents rapports pondéraux de IgG/IgG-^{125}I. (b) Inhibition avec l'HSA non marquée. Des quantités croissantes de HSA non marquée ont été ajoutées à 150 µg d'IgG-^{125}I ce mélange est ensuite incubé avec 8,1 10^{8} érythrocytes. La quantité d'IgG-^{125}I fixée aux érythrocytes est exprimée en µg par culot pour différents rapports pondéraux de HSA/IgG-^{125}I. Les témoins représentent la quantité d'IgG-^{125}I fixée aux érythrocytes sans adjonction de protéines non marquées et permettent de calculer le pourcentage d'inhibition produit par l'adjonction d'IgG ou d'HSA non marquées.

de l'IgG non traitée sont également mesurés. Un témoin mesure la fixation cellulaire de l IgG-^{125}I sans adjonction d IgG non marquée. Les résultats sont exprimés sur la figure 2. On voit que la fixation de l'IgG-^{125}I aux érythrocytes n est significativement diminuée que par l adjonction d IgG exposée à pH 2,9

Inhibition par l'hydrolysat pepsique de l'IgG Des quantités croissantes d un hydrolysat pepsique de l'IgG sont ajoutées à une quantité constante d'IgG-^{125}I La fixation de l IgG-^{125}I aux culots d'érythrocytes est comparée à celle d'un contrôle sans adjonction d'hydrolysat pepsique de l'IgG L effet inhibiteur d une même quantité d'IgG non digérée est aussi mesuré (tableau I) On constate que l adjonction d une quantité d'hydrolysat pepsique de l IgG égale, sur une base pondérale, à celle de l IgG-^{125}I, produit une diminution significative de la fixation de l IgG-^{125}I aux érythrocytes. Cette diminution n est pas plus marquée en ajoutant 10 fois plus d'hydrolysat pepsique de l'IgG

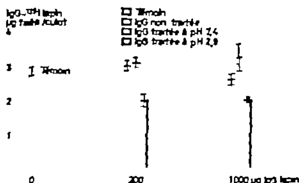


Fig. 2. Inhibition de la fixation de l'IgG-125I aux érythrocytes par l'IgG traitée à pH 2,9 et réajustée à pH 7,4. Une quantité de 200 µg d'IgG-125I a été ajoutée à 200 µg ou 1000 µg d'IgG non traitée, d'IgG traitée à pH 7,4 ou d'IgG traitée à pH 2,9. Ces différents mélanges sont incubés avec 32,4 · 10⁸ érythrocytes. On compare la quantité d'IgG-125I fixée en µg par culot dans les différentes séries. Les témoins représentent la quantité d'IgG-125I fixée aux érythrocytes sans adjonction d'IgG non marquée. Chaque point représente la moyenne de 5 valeurs ± l'erreur moyenne de la moyenne ($\bar{x} \pm SE$). Différence statistiquement significative.

Tableau I. Inhibition de la fixation de l'IgG-125I aux érythrocytes par l'adjonction d'un hydrolysat pepsique de l'IgG

	µg d'inhibiteur	µg d'IgG-125I fixée/culot
Contrôle	–	1,35 ± 0,16[1]
Hydrolysat pepsique de l'IgG	100	0,78 ± 0,05 $p < 0,02$[1]
	1 000	0,71 ± 0,07 $p < 0,01$
IgG non digérée	1 000	0,99 ± 0,09[2] NS[3]

100 µg d'IgG-125I ont été mélangés à 100 µg ou 1000 µg d'hydrolysat pepsique de l'IgG ou encore à 1000 µg d'IgG non digérée. Ces différents mélanges ont été incubés avec 32,4 · 10⁸ érythrocytes. La quantité d'IgG-125I fixée aux différents culots est comparée à celle obtenue avec le contrôle sans adjonction d'inhibiteur. Valeurs $\bar{x}$ ± SE. La valeur de p indique une différence statistiquement significative.

Nombre d'essais $n = 5$.
Nombre d'essais $n = 4$.
Différence statistiquement non significative.

Inhibition par différents fragments de l'IgG. Afin de rendre plus sensible le système d'inhibition, on utilise dans cette expérience une faible quantité d'IgG-125I à laquelle sont ajoutés les fragments Fc et Fab en quantité molaire 10 fois supérieure à celle de l'IgG-125I. On admet que

Tableau II Comparaison de différents inhibiteurs de la fixation de l'IgG-^{125}I aux érythrocytes

	µg d'inhibiteur	µg d'IgG-^{125}I fixés/culot	
Contrôle		1,03 ± 0,07	
Fragment Fab	166	0,72 ± 0,04 $p < 0,01$	
			$p < 0,05$
Fragment F	166	0,50 ± 0,06 $p < 0,001$	
Fab + F	166	0,67 ± 0,08 $p < 0,01$	
Hydrolysat pepsique de l'IgG non dialysée	500	0,49 ± 0,04 $p < 0,001$	
			p = NS
Hydrolysat pepsique de l'IgG dialysée	500	0,42 ± 0,06 $p < 0,001$	
IgG	500	0,65 ± 0,03 $p < 0,01$	

30 µg d'IgG-^{125}I ont été incubés avec 32,4 10^6 érythrocytes en présence ou non d'inhibiteur Valeurs $\bar{x}$ ± SE nombre d'essais = 3. La valeur de p indique une différence statistiquement significative par rapport au contrôle. La valeur de p indique une différence statistiquement significative entre deux inhibiteurs considérés. NS = différence statistiquement non significative.

le Fc et le Fab ont un poids moléculaire égal environ au ⅓ de celui de l IgG L effet inhibiteur de l IgG native en quantité équimolaire aux fragments de l IgG est également mesuré. De plus, on étudie le pouvoir inhibiteur de l'hydrolysat pepsique de l'IgG avant et après dialyse (tableau II) Ces résultats montrent que tous les inhibiteurs ajoutés en quantité molaire 10 fois supérieure à l IgG-^{125}I ont un effet inhibiteur statistiquement significatif par rapport au contrôle. En outre, l'effet inhibiteur du fragment Fc est significativement plus important que celui du fragment Fab Par contre, il n y a pas de différence significative entre l effet inhibiteur de l'hydrolysat de l'IgG dialysé ou non dialysé. De ce fait, les peptides dialysables ne semblent pas jouer de rôle dans le pouvoir inhibiteur de l'hydrolysat pepsique de l'IgG

Inhibition par le sérum humain normal (SHN) et par le SHN absorbé sur des érythrocytes de lapin L effet inhibiteur du SHN a été étudié et comparé à celui du SHN absorbé 3 fois sur des érythrocytes de lapin (tableau III) Les résultats montrent que le facteur sérique inhibiteur de la fixation de l'IgG-^{125}I aux érythrocytes n est pas épuisé par trois absorptions successives sur les érythrocytes de lapin.

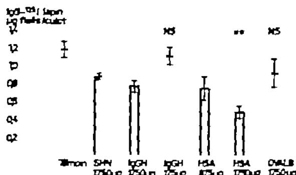


Fig. 3. Inhibition de la fixation de l'IgG-^{125}I aux érythrocytes par le SHN, par l'IgG humaine, par l'HSA et par l'ovalbumine. Une quantité de 175 µg d'IgG-^{125}I est mélangée à une quantité 10 fois plus grande de SHN, d'IgG humaine, d'HSA ou d'ovalbumine. Ces mélanges sont incubés avec 32,4 10^6 érythrocytes. L'effet inhibiteur de l'IgG ou de l'HSA à des concentrations sériques est aussi étudié. Le témoin représente la quantité d'IgG-^{125}I fixée aux érythrocytes sans adjonction d'inhibiteur. On compare la quantité d'IgG-^{125}I fixée aux érythrocytes des différentes séries à celle obtenue avec le contrôle. Chaque point représente la moyenne de 5 valeurs ± l'erreur moyenne de la moyenne ($\bar{x} \pm SE$). Différence statistiquement significative ($p < 0,05$); NS = Différence statistiquement non significative.

Tableau III. Inhibition par le SHN et par le SHN absorbé sur des érythrocytes

µg de protéines sériques ajoutés		µg d'IgG-^{125}I fixés/culot	µg d'IgG-^{125}I fixés sur le verre
Contrôle	–	0,82 ± 0,09	0,91 ± 0,08
SHN absorbé	1750	0,31 ± 0,06 $p < 0,02$	0,38 ± 0,03 $p < 0,001$
		p = NS	p = NS
SHN non absorbé	1750	0,50 ± 0,04 $p < 0,02$	0,38 ± 0,02 $p < 0,001$

400 µg d'IgG-^{125}I en présence de SHN absorbé ou non absorbé ont été incubés avec 32,4 10^6 érythrocytes. Valeurs ¯ ± SE nombre d'essais n = 5. La valeur de p indique une différence statistiquement significative par rapport au contrôle. La valeur de p' indique une différence statistiquement non significative entre le SHN absorbé et non absorbé.

Enfin, on compare l'effet inhibiteur du SHN de l'IgG humaine, de l'HSA et de l'ovalbumine sur la fixation de l'IgG aux érythrocytes (fig. 3). Les quantités d'inhibiteur utilisées sont égales à 10 fois celle de l'IgG-^{125}I. L'effet inhibiteur d'une quantité d'IgG (175 µg) ou d'HSA (875 µg) égale à celle contenue dans 1750 µg de protéines sériques est également étudié. On constate que l'IgG et l'ovalbumine

n ont d effet inhibiteur qu à une quantité 10 fois supérieure à celle de l'IgG-^{125}I Le même effet inhibiteur est obtenu avec 1750 μg de SHN qu avec la quantité d HSA seule (875 μg) contenue dans 1750 μg de SHN Par contre, la quantité d IgG (175 μg) contenue dans 1750 μg de SHN n a pas d effet inhibiteur significatif.

Déplacement de l'IgG fixée aux érythrocytes par réincubation avec des protéines non marquées. L expérience suivante démontre le déplacement de l'IgG-^{125}I fixée aux érythrocytes par l adjonction d'IgG ou d'HSA lors d une réincubation. Un contrôle est fait en remplaçant l adjonction de protéines non marquées par du tampon. La quantité d'IgG-^{125}I fixée aux érythrocytes à la première incubation est comparée à celle mesurée après la deuxième incubation. Parallèlement, on mesure la quantité d IgG-^{125}I présente dans le surnageant du dernier lavage, avant et après la réincubation. Dans ces expériences, la fixation de l IgG-^{125}I sur le verre est incluse dans les valeurs obtenues pour le culot. Les résultats montrent que le déplacement de l IgG-^{125}I fixée aux érythrocytes avec l'IgG ou l HSA non marquées est négligeable et ne se distingue pas de façon significative de l effet de dilution et d'élution du tampon dans la série de contrôle (tableau IV)

Enfin, on étudie le déplacement de l IgG-^{125}I fixée aux érythrocytes produit par la réincubation avec une quantité molaire d'IgG ou

Tableau IV Déplacement de l'IgG-^{125}I fixée aux érythrocytes au cours d'une réincubation d'IgG ou d'HSA non marquées

	Première incubation avec IgG-^{125}I		Deuxième incubation (déplacement)		
	μg d'IgG-^{125}I fixée/culot	μg d'IgG-^{125}I dans le surnageant		μg d'IgG-^{125}I fixée/culot	μg d'IgG-^{125}I dans le surnageant
I	2,66 ± 0,25[1]	0,82 ± 0,07[1]	I avec tampon (contrôle)	2,01 ± 0,13	1,89 ± 0,17
II	2,70 ± 0,16	0,98 ± 0,08	II avec IgG	1,93 ± 0,18 NS	1,74 ± 0,16 NS
III	2,73 ± 0,11	0,89 ± 0,08	III avec HSA	2,10 ± 0,13 NS	1,66 ± 0,15 NS

Pour les trois séries (I II, III) la première incubation été faite en présence de 200 μg d'IgG-^{125}I et 22,4 10^{6} érythrocytes. La deuxième incubation (déplacement) été faite en joutant aux culots de la première incubation 200 μg d'IgG ou d'HSA, ou encore un volume égal de tampon ordinaire pour la série de contrôle. Les lavages ont été faits avec une solution isotonique de glucose, tamponnée avec le tampon ordinaire. Valeurs ‾ ± SE, nombre d'essais n = 5.

Nombre d'essais n = 6. NS = différence statistiquement non significative.

Tableau V. Déplacement de l'IgG-^{125}I fixée aux érythrocytes au cours d'une réincubation avec de l'IgG ou de l'HSA non marquées en grand excès

Première incubation avec IgG-^{125}I		Deuxième incubation (déplacement)		
µg d'IgG-^{125}I fixés/culot	µg d'IgG-^{125}I dans le surnageant		µg d'IgG-^{125}I fixés/culot	µg d'IgG-^{125}I dans le surnageant
I 6,16 ± 0,30	1,65 ± 0,03	I avec tampon (contrôle)	4,03 ± 0,34	2,61 ± 0,17
II 6,39 ± 0,09	1,39 ± 0,12	II avec IgG	2,87 ± 0,12 $p < 0,001$	4,37 ± 0,13 $p < 0,001$
III 6,90 ± 0,43	1,60 ± 0,10	III avec HSA	4,20 ± 0,20 NS	3,01 ± 0,03 NS

Pour les 3 séries (I, II, III), la première incubation a été faite en présence de 400 µg d'IgG-^{125}I et 32,4 · 10^{8} érythrocytes. La deuxième incubation (déplacement) a été faite en ajoutant aux culots de la première incubation 4000 µg d'IgG ou d'HSA, ou encore un volume égal de tampon ordinaire pour la série contrôle. Les lavages ont été faits avec une solution de glucose isotonique tamponnée avec le tampon ordinaire. Valeurs $\bar{x} \pm SE$; nombre d'essais n = 5. La valeur de p indique une différence statistiquement significative, par rapport au contrôle. NS = différence statistiquement non significative.

d'HSA 10 fois supérieure à celle de l'IgG-^{125}I introduite lors de la première incubation (tableau V). Dans ces conditions, l'IgG non marquée déplace, significativement par rapport au contrôle, l'IgG-^{125}I fixée aux érythrocytes. Ceci est indiqué, lors de la réincubation, par une diminution de la radioactivité du culot et une augmentation de la radioactivité dans le surnageant. Ce déplacement n'a pas lieu de façon statistiquement significative avec une quantité molaire égale d'HSA non marquée.

Discussion

Nos résultats montrent que la fixation non spécifique de l'IgG aux érythrocytes ne peut être diminuée qu'en présence d'un grand excès d'inhibiteur. Il en est de même pour l'inhibition de la fixation spécifique par un inhibiteur non spécifique [5]. Le fait que la membrane érythrocytaire n'est pas saturée par une quantité bien définie d'IgG diminue la sensibilité du système d'inhibition. L'albumine est un meilleur inhibiteur que l'IgG ce qui pourrait s'expliquer par une différence d'hydratation moléculaire de ces deux protéines. L'albumine diminuerait les liaisons hydrophobes impliquées dans la fixation

non spécifique de l'IgG L effet inhibiteur du sérum humain normal serait dû à sa forte concentration en albumine, non épuisée par trois absorptions sur des érythrocytes l existence d autres facteurs sériques inhibiteurs ne peut pourtant pas être exclue. Il faut noter que l'HSA ^{125}I ne se fixe pas plus que l IgG-^{125}I sur les érythrocytes. Ainsi l efficacité de l inhibiteur n est pas nécessairement liée à la fixation cellulaire de l inhibiteur comme le suggère BENACERRAF [1] De même, on observe que l'hydrolysat pepsique de l IgG est plus inhibiteur qu une même quantité, sur une base pondérale, d'IgG native. Les travaux d'ISLIKER *et al* [6] montrent que les γG-globulines digérées par la pepsine, donc dépourvues du fragment Fc, ne se fixent plus non spécifiquement aux hépatocytes. Dans des conditions expérimentales favorisant l'inhibiteur nos résultats indiquent que le fragment Fc est plus inhibiteur que le fragment Fab les travaux de GROS *et al* [4] montrent, par ailleurs, que la fixation cellulaire de ces deux fragments n est pas différente.

Après la fixation aux érythrocytes, le déplacement des molécules d'IgG-^{125}I est minime et nécessite une réincubation avec un grand excès d IgG L ensemble de ces résultats ne permet pas de tirer des conclusions sur l existence d un site de fixation bien défini sur la molécule d IgG d une part ou sur la membrane érythrocytaire d'autre part. La présence d'un site de fixation non spécifique sur la membrane cellulaire impliquerait que la quantité d'IgG fixée soit directement proportionnelle au nombre d érythrocytes ou à la surface cellulaire disponible, ce qui n est pas observé dans nos expériences. De plus, il n existe pas de parallélisme entre l'effet inhibiteur et la capacité de fixation cellulaire de l'inhibiteur suggérant que le processus d inhibition n est pas nécessairement situé au niveau d un site de fixation.

On peut conclure que les molécules d'IgG se fixeraient à la surface de la membrane sans une orientation moléculaire définie. Ceci permettrait d'expliquer l'inhibition peu importante par les différentes protéines considérées. En effet, une orientation non définie de la molécule d IgG fixée non spécifiquement et la grande capacité de fixation de la membrane érythrocytaire diminuent l'influence d un inhibiteur L étude *in vitro* de l'inhibition de la fixation non spécifique de l'IgG aux érythrocytes nécessiterait d établir dans quelle mesure ces observations reflètent les processus biologiques *in vivo*. Les conséquences et le mécanisme d une inhibition *in vivo* telle qu elle a été décrite chez un patient par JEANNET et coll. [7] doivent encore être éclaircis.

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Résumé

Différents inhibiteurs de la fixation non spécifique de l'IgG aux érythrocytes ont été étudiés. L inhibition de la fixation non spécifique est peu importante et nécessite un grand excès d'inhibiteur L effet d'inhibition n est pas nécessairement dû à la fixation de l'inhibiteur aux érythrocytes. Les inhibiteurs les plus efficaces sont l'albumine et le sérum humain. Dans des conditions favorisant l'inhibition, le fragment Fc est plus inhibiteur que le fragment Fab. Les molécules d'IgG pourraient se fixer non spécifiquement aux érythrocytes sans une orientation moléculaire bien définie.

Summary

Inhibition of the process of fixation is not readily accomplished and fixation of ^{125}I-IgG is significantly inhibited only with large excess of inhibitor Inhibition is not necessarily due to fixation of the inhibitor to erythrocytes. The most potent inhibitors are human serum albumin and human serum. In conditions favorable to inhibition, F fragment is more active than F b fragment. The IgG molecules appear to be non specifically fixed to erythrocytes without any particular orientation.

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Adresse des auteurs: Dr Dominique des Gouttes et Prof. Dr H. Isliker, Institut de Biochimie, Université de Lausanne, 1000 Lausanne (Suisse).

Tabelle I. Anteil Immunglobulinhaltiger Zellen in Leukozytenkonzentraten aus dem peripheren Blut von Patienten mit verschiedenen Formen von Hepatitis

Fall Nr	Ig	kl. Ly in %	m. Ly in %	gr. Ly in %	Plz. in %	gesamt in ‰	davon Plz. in ‰	abs. MN	Plz. in %
1	γG	–	+	–	+	< 1		–	–
	γA					negativ			
	γM								
2	12. 11. 1968								
	γG	25,2	49,4	5,7	18,2	14,6	2,7	2 775	13
	γA	24,0	79,2	9,6	8,4	3,5	0,14		
	γM	–	+	–	–	< 1			
	20. 11. 1968								
	γG	4,6	64,8	14,0	16,7	18,5	3,1	2 448	2,1
	γA					nicht untersucht			
	γM								
3	γG	37,0	52,8	6,6	2,2	8,7	0,13	2 250	4,3
	γA	+	+	–	–	< 1			
	γM	+	+	–	–	< 1			
4	γG	81,4	16,8	–	3,1	6,0	0,75	–	–
	γA	+	+	–	–	< 1			
	γM	+	+	–	–	< 1			
5	γG	32,7	45,5	8,6	14,4	6,8	1,0	–	–
	γA	44,0	22,0	–	33,0	3,0	1,0		
	γM	+	+	–	+	1			
6	γG	20,0	57,5	10,5	15,0	5,0	0,75	6 040	–
	γA	+	+	–	+	< 1			
	γM	–	+	–	–	< 1			

kl. Ly = kleine Lymphozyten, m. Ly = mittlere Lymphozyten, gr. Ly = grosse Lymphozyten, Plz. = Plasmazellen, abs. MN = nach dem Differentialblutbild errechnete Anzahl von mononukleären Zellen/mm³, + = immunzytologisch positive Zellen vorhanden.

Ergebnisse

Tabelle I zeigt den prozentuellen und absoluten Anteil Immunglobulinhaltiger Zellen in Leukozytenkonzentraten aus dem peripheren Blut einer Normalperson (Fall 1) und von Patienten mit verschiedenen Formen von Hepatitis. Fall 2–4 zeigen klinisch das Bild einer unkomplizierten Hepatitis, die Leukozytenabnahme erfolgt innerhalb der ersten 10 Tage nach Auftreten eines Ikterus. Bei Fall

Tabelle II. Immunglobulinhaltige Zellen in Leukozytenkonzentraten von Patienten mit Agranulozytose und Mononucleosis infectiosa

Fall Nr	Ig	kl. Ly in %	m. Ly in %	gr Ly in %	Plz. in %	gesamt in ‰	davon Plz. in ‰	abs. MN	Plz. in %
7	28.10.1968								
	γG	nicht differenziert				8,3	1	921	–
	γA	+	+	–	–	1			
	γM	+	+	–	–	0,9			
	4.11.1968								
	γG	16,8	73,2	7,2	2,4	8,4	0,2	3 025	1 7
	γA	26,4	52,8	–	19,8	1,5	0,3		
	γM					negativ			
8	γG	monozytoide Reizformen, keine Plz.				3,5		–	–
	γA					nicht untersucht			
	γM								
9	γG	+	+	–	+	< 1		2 520	–
	γA	+	–	–	–	1,0			
	γM					negativ			

kl. Ly = kleine Lymphozyten m. Ly = mittlere Lymphozyten; gr Ly = grosse Lymphozyten; Plz. = Plasmazellen; abs. MN = nach dem Differentialblutbild errechnete Anzahl von mononukleären Zellen/mm³ + = immunzytologisch positive Zellen vorhanden.

5 wurde histologisch eine aktive, chronische Hepatitis, bei Fall 6 eine granulomatöse Hepatitis ungeklärter Ätiologie diagnostiziert. Auffällig und in seiner Bedeutung bisher ungeklärt ist der relativ hohe Anteil von γA haltigen Zellen bei Fall 2 und 5 der prozentuelle Plasmazellanteil scheint parallel der absoluten Anzahl positiver mononukleärer Zellen anzusteigen. Ebenfalls nur beschrieben werden kann derzeit der stark wechselnde Anteil kleiner bzw mittlerer Lymphozyten Wurden bei der Auszählung von γA oder γM-haltigen Zellen weniger als 1 ‰ positiver Zellen gefunden, so sind in der Tabelle lediglich der vorherrschende Zelltyp, meist kleine bis mittlere Lymphozyten, vermerkt.

In Tabelle II ist der Anteil Immunglobulinhaltiger Zellen in Leukozytenkonzentraten einer Patientin mit medikamentös bedingter Agranulozytose (Fall 7) sowie zweier Patienten mit Mononucleosis infectiosa (Fall 8 und 9) aufgeführt. Fall 8 befand sich im akuten Stadium, alle γG-positiven Zellen imponierten monozytoid. Fall 9 kam 6 Monate nach einer Erkrankung an infektiöser Mononukleose

Tabelle III. Immunglobulinhaltige Zellen in Leukozytenkonzentraten von Patienten mit M. Hodgkin, chronischer Lymphadenose, γG-Plasmozytom und M. Waldenström

Fall Nr.	Ig	kl.Ly in %	m.Ly in %	gr Ly in %	Plz. in %	gesamt in ‰	davon Plz. in ‰	abs. MN	Plz. in %
10	γG	+	+	–	–	< 1	–	1 280	–
	γA					negativ			
	γM								
11	γG							240 000	
	γA					negativ			
	γM								
12	19.4.1969								
	γG		39,6	13,2	46,2	5,8	2,7	1 310	2,8
	γA					negativ			
	γM	+	+	–	–	< 1			
	κ		35,0	21,0	42,0	7,0	2,9		
	24.4.1969								
	γG		87	6,0	6,0	11,1	0,8	4 840	–
	γA	+	+	–	–	1,5	–		
	γM					negativ			
	κ		84,0	8,0	6,6	12,3	0,8		
13	19.4.1969								
	γG	–	+	–	–	< 1	–	3 870	–
	γA	–	+	–	–	< 1	–		
	γM	+	+	+	+	etwa 1%			
	κ	analoge Zellpopulation positiv				etwa 1%			
	24.4.1969	unverändertes Zellbild, analoge Anzahl positiver Zellen.							

Kleine und mittlere Lymphozyten zusammen.
kl.Ly = kleine Lymphozyten m.Ly = mittlere Lymphozyten gr Ly = grosse Lymphozyten Plz. = Plasmazellen abs.MN = nach dem Differentialblutbild errechnete Anzahl von mononukleären Zellen/mm³ + = immunzytologisch positive Zellen vorhanden.

beschwerdefrei zu einer Kontrolluntersuchung die keinen krankhaften Befund erbrachte Auch hier ist die Bedeutung der stark positiven γA haltigen Lymphozyten ungeklärt neben den sorgfältigen Spezifitätskontrollen weisen immerhin auch diese unterschiedlich positiven Zellklassen auf die Spezifität des immunzytologischen Nachweises hin.

In Tabelle III wurden die Befunde von 4 Patienten mit malignen hämatologischen Erkrankungen zusammengefasst. Auffällig ist der spärliche bis negative Befund bei Fall 10, einem Patienten mit M Hodgkin und Fall 11 einem mit chronischer Lymphadenose. Hier er

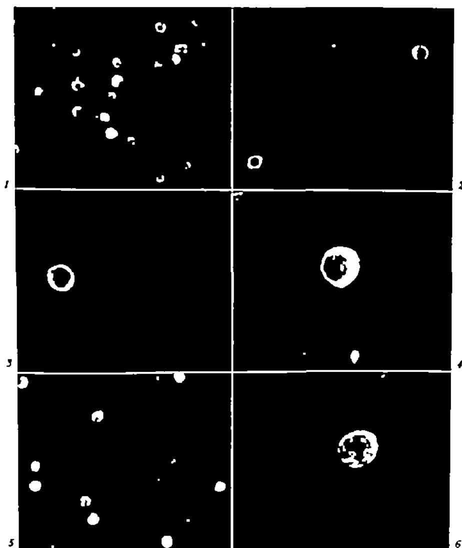


Abb. 1 Testknochenmarkausstrich eines Patienten mit γA-Plasmocytom. Zahlreiche γA-positive Plasmazellen (200)
Abb. 2. Zwei kleine periphere γA-positive Lymphocyten. Fall 9 Status nach Mononucleosis infectiosa (250)
Abb. 3. Mittelgroßer γG-positiver Lymphocyt Fall 3 akute Hepatitis (400)
Abb. 4 Periphere γM-positive Plasmazelle. Fall 5 chronische Hepatitis (630)
Abb. 5. Zahlreiche κ-positive lymphoide Zellen in einem Leukocytenkonzentrat. Fall 13 M. Waldenström (200)
Abb. 6. γM-positiver kleiner bis mittelgroßer Lymphocyt. Fall 13 M. Waldenström (630)



Abb. 7 Kleiner bis mittelgrosser κ-positiver Lymphozyt. Fall 13 M. Waldenström (×630).
Abb. 8 Grosser γM-positiver Lymphozyt. Fall 13: M. Waldenström (630)
Abb. 9 κ-positiver grosser Lymphozyt. Fall 12 γG-Plasmozytom vom κ Typ (×630)
Abb. 10 Grosser γG-positiver Lymphozyt. Fall 7 medikamentös bedingte Agranulozytose in Remission (×630)

geben sich Parallelen zu dem bei diesen Erkrankungen beschriebenen Antikörpermangelsyndrom Fall 12 zeigt die Werte eines Patienten mit γG-Plasmozytom vom κ Typ Gegenüber allen anderen untersuchten Fällen fand sich hierbei im unbehandelten Zustand ein deutlich höherer Prozentsatz von Plasmazellen der allerdings nach nur kurzer zytostatischer Therapie trotz Zunahme der Gesamtzahl von positiven Zellen zugunsten kleiner Lymphozyten auf 6 abfiel. Eine analoge Zellpopulation erwies sich als γG- und κ-positiv auffälligerweise auch alle Grössenordnungen von Lymphozyten so dass man sie wohl als ausgeschwemmte Tumorzellen zu betrachten hat. Bei Fall 13 handelt es sich um eine unbehandelte Patientin mit M. Waldenström, die ebenfalls einen relativ hohen Prozentsatz Paraproteinhaltiger mononukleärer Zellen in der Peripherie aufwies.

Diskussion

Die Frage, ob Plasmazellen aus Lymphozyten entstehen können ist noch immer nicht endgültig geklärt. In Tierversuchen konnte gezeigt werden dass nach Immunisierung ein kleiner Teil der peripheren Leukozyten Antikörper bilden kann [5 7 9 12] Die morphologische Einordnung dieser Population stösst allerdings auf Schwierigkeiten. Der elektronenmikroskopische Nachweis von endoplasmatischem Reticulum als Ausdruck einer Antikörperbildung konnte lange Zeit lediglich für Plasmazellen erbracht werden Erst in letzter Zeit wurden Befunde erhoben, die für eine Entwicklung ruhender stoffwechsel

inaktiver Lymphozyten über basophile «Blasten» mit spärlichen Proteinsyntheseorganellen in vollentwickelte Plasmazellen sprechen [3 4 7 8, 11]

Unsere Untersuchungen bestätigen, dass bereits bei Normalpersonen eine allerdings sehr kleine Zahl von peripheren, mittelgrossen Lymphozyten Immunglobuline enthalten [13] Bei Erkrankungen mit peripheren «lymphatischen Reizformen» Virusinfekten und allergischen Reaktionen erhöht sich die Zahl dieser Zellen.

Ein gesetzmässiges Auftreten der einzelnen Zellklassen im Rahmen eines Krankheitsverlaufes ist bei den angeführten Fällen nicht zu sichern immerhin ist der Anteil grosser Lymphozyten gegenüber dem kleiner und mittlerer relativ konstant [3 4] Es fiel weiterhin auf, dass die grossen Zellen mit häufigen Mitosen bei den Fällen mit Paraproteinämie etwas öfter «κ positiv» als γG- bzw γM positiv waren Dies ist möglicherweise der Ausdruck eines besonders aktiven intrazellulären «light-chain-pools» wie er bei Paraproteinämien mit Bence-Jones-Proteinurie beschrieben wurde [2]

Soweit diese Methode absolute Angaben zulässt, schwankte die Zahl der Iz-positiven Zellen zwischen weniger als 1 /₀₀ und etwa 20°/₀₀. Bei einem Fall mit Morbus Waldenström waren 1% der mononukleären Zellen – alle Grössenordnungen von Lymphozyten, plasmoiden Zellen und Plasmazellen – peripher iz positiv

Die drei Ig Klassen konnten im Zytoplasma aller lymphatischen Zellklassen gefunden werden, auch in eindeutig sehr kleinen Lymphozyten Van Furth konnte in diesen Zellen nur γM nachweisen [13] Die Erklärung dürfte in der Verwendung unterschiedlich starker Antiseren liegen.

Der Vergleich der iz positiven Plasmazellen mit dem nach morphologischen Kriterien errechneten Plasmazellanteil zeigte einen deutlich niedrigeren Wert. Diese schlechte Korrelation dürfte mit dem Verlust von Plasmazellen während der Präparation zusammenhängen Es war schon bei früheren Untersuchungen aufgefallen, dass trotz eines 4–5%igen Anteiles von Plasmazellen im Differentialblutbild im Leukozytenkonzentrat manchmal nur mehr spärlich Plasmazellen zu finden waren. Die angegebenen Absolutzahlen der iz-positiven Plasmazellen liegen also sicher zu niedrig Eine enge Beziehung zwischen Anzahl der «lymphatischen Reizformen» und iz positiven Lymphozyten ist nicht zu erwarten, da nur ein kleiner Teil der stoffwechselaktiven Zellen Ig synthetisieren [3 u. a.]

Versuche, die κ-positiven Zellen mit Ergebnissen der Methylgrünpyronin Färbung zu korrelieren zeigte eine ähnlich grosse Anzahl mittlerer und grosser Lymphozyten mit stark pyroninophilem Zytoplasma, obwohl diese Farbintensitätsunterschiede schwer zu objektivieren sind.

Aufschlussreich ist die Anzahl der positiven Zellen nach «Färbung» mit fluoreszierendem Anti κ-Serum bei den beiden Fällen mit γG-Plasmozytom und M. Waldenström vom κ Typ Es darf als gesichert gelten dass eine Zelle jeweils nur einen Typ von Ig bildet, entweder Globuline mit leichten Ketten vom κ Typ oder λ Typ [14 u.a.] Van Furth [14] konnte in einer Untersuchung über den prozentuellen Anteil von κ und λ produzierenden Zellen im Knochenmark für Normalpersonen ein Verhältnis von 3 1 in der Peripherie von 3 5 1 ermitteln und diese Befunde mit der *in vitro de novo*-Synthese von κ und λ antigenen Globulinen korrelieren. In Knochenmarkspräparationen von Patienten mit Plasmozytom vom κ Typ waren 99% der Plasmazellen κ positiv Bei benignen monoklonalen Paraproteinämien betrug das Verhältnis der κ λ positiven Zellen im Knochenmark 11 2 1 während in der Peripherie keine wesentliche Veränderung gegenüber Normalpersonen beobachtet werden konnte (3,5 1) da in diesen Fällen offenbar das Hauptkontingent von Ig haltigen Zellen aus lymphatischen Organen stammt. Der Anteil κ positiver Zellen in der Peripherie von Patienten mit γG Plasmozytom vom κ Typ wird nicht mitgeteilt. Die Auszählung der κ positiven peripheren Zellen bei unseren beiden Fällen zeigte, daß die überwiegende Zahl, aber nicht nur Plasmazellen sondern auch kleine und mittlere Lymphozyten das jeweilige Paraprotein im Zytoplasma enthielten und eine abzüglich der methodischen Fehlerbreite gleich grosse Anzahl und analoge Zellpopulation vom κ Typ waren. Es liegt nahe, alle Zellen die bei einem monoklonalen Plasmozytom an der Paraproteinbildung teilhaben als intermediäre Formen im Rahmen der Entwicklung von Plasmazellen zu werten.

Zusammenfassung

Es wurde bei 12 Patienten mit lymphatischen Reizformen im Differentialblutbild immunzytologisch Immunglobuline vom γG- γA und γM-Typ im Zytoplasma kleiner mittlerer und grosser Lymphozyten und Plasmazellen nachgewiesen. Der prozentuelle Anteil der einzelnen positiven Zellklassen wies bei den kleinen und mittleren Lymphozyten sowie bei den Plasmazellen erhebliche Schwankungen auf, während der Anteil grosser

Lymphozyten relativ konstant um 10% lag. Die absolute Anzahl positiver Zellen schwankte zwischen weniger als 1 $‰$ und 1 $\%$ der mononukleären Zellen in den Leukozytenkonzentraten. Bei 2 unbehandelten Patienten mit κ Typ-Paraproteinämien (γG-Plasmozytom, M. Waldenström) enthielten annähernd 100% der immunzytologisch positiven Zellen in der Peripherie das Paraprotein, welches noch zusätzlich durch seine κ-Eigenschaft charakterisiert wurde.

Summary

Immune globulins of the gamma-G, gamma-A and gamma-M types were found in the cytoplasm of small, medium and large lymphocytes and plasmacytes of 12 patients presenting signs of lymphatic irritation in the differential blood count. The percentage of positives varied widely in the small and medium lymphocytes and the plasma cells, but was relatively constant (about 10%) in the large lymphocytes. Absolute numbers of positive cells ranged from less than 0.1% up to 1% of the mononuclear cells in the leukocyte concentrates. In two untreated patients with kappa-type paraproteinaemia (gamma-G plasmocytoma, M. Waldenström) almost 100% of the immunologically positive cells in the peripheral blood contained the paraprotein, which was further characterized by its kappa property

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Adresse der Autoren: Dr. H. Asamer und Prof. Dr. H. Braunsteiner, Medizinische Universitätsklinik, *Innsbruck* (Österreich)

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Department of Medicine University of Aberdeen, Aberdeen

Exercise-Induced Thrombocytosis

A. A. DAWSON and D OGSTON

Most investigators have reported that brisk exercise of short duration induces a transient rise in the platelet count [3 10, 11] but there is not universal agreement on this [4] The mechanism of the thrombocytosis in response to exercise is unknown We report a study on the effect of exercise on the platelet count in healthy men and women and in asplenic subjects, and on a comparison of the platelet response to exercise before and after β-adrenergic blockade.

Methods and Subjects

Platelet counts were performed by the method of [illegible] and [illegible] [8] on venous blood samples anticoagulated with disodium ethylenediamine-tetraacetate (EDTA) The normal range for healthy subjects in this laboratory is 150–270,000/mm³ It was calculated from the results of 30 duplicate blood samples with platelet counts ranging from 117,000 to 534,000/mm³ that the difference between duplicates, as percentage of each mean platelet count, had mean value of 10.8% (SD ± 6.8%)

Subjects. Twelve men and 6 women aged 18 to 36 years and in good health participated in the experiments. In addition, 4 men and 2 women aged 17 to 29 who had undergone splenectomy 6 months to 6 years previously were studied splenectomy had been performed as result of trauma in 4 of these subjects, because of spontaneous rupture during infectious mononucleosis in one, and for the treatment of hemolytic anemia in the remaining subjec All were in good health t the time of the study

Exercise was undertaken on an electrically braked bicycle ergometer (Lode Instrumenten N V Groningen, Holland) Work loads are expressed in metre-kiloponds (mkp) The procedure took place at 9.30 a.m. or 2.15 p.m. After the subject rested for 15 min the initial blood sample was withdrawn. Further samples were obtained after 5 and 15 minutes' exercise and then after resting for 5 and 15 min.

Propranolol (Inderal®) was taken orally 60 mg in divided doses during the 24 h preceding the exercise, 20 mg being taken 1 h before the test.

Table I. Effect f exercise on the platelet count of healthy men and women

Exercise load mkp	Sex	No. in group	Platelet counts × $10^3/mm^3$ (means ± SD)				
			Before exercise	Minutes after start of exercise		Minutes after end of exercise	
				5	15	5	15
600	F	6	185±54	223±34	239±61	211±40	189±39
600	M	6	184±36	174±31	169±24	174±32	170±33
900	M	6	166±41	199±48	224±31	203±52	213±49

Results

Effect of exercise on the platelet count of healthy men and women. Six men and 6 women completed exercise at a work load of 600 mkp for a duration of 15 min and a further 6 men completed this period of exercise at a work load of 900 mkp. The mean platelet counts before during and after exercise are presented in table I. At a work load of 600 mkp there was a significant rise in the platelet count in women after 15 minutes exercise ($p < 0.01$). In men there was no significant change in the platelet count ($p > 0.1$). The increase in the platelet count in men at a work load of 900 mkp was significant both at 5 min (p 0.05) and at 15 min ($p < 0.01$). In contrast to the women, the platelet count of these men had not returned to the initial level after 15 minutes rest. The hematocrit changes during exercise were small or absent and could not account for the alterations in the platelet count.

Effect of propranolol on the rise in the platelet count induced by exercise. Three men and 4 women who had exercised at 900 mkp and 600 mkp respectively were retested at least 1 week later after receiving oral propranolol. The mean pulse-rate after exercise for 15 min fell from 13? min without propranolol to 102/min when propranolol was given before exercise this suggests that propranolol had produced significant β-adrenergic blockage. The individual changes in the platelet count with and without prior propranolol are shown in figure 1. The mean rise in th platelet count with and without propranolol did not differ significantly ($p > 0.1$).

Effect of exercise on the platelet count in asplenic subjects. Four men and 2 women who had previously undergone splenectomy exercised at a

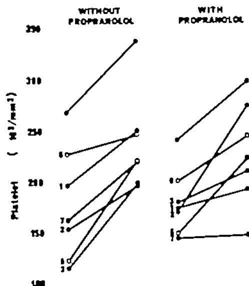
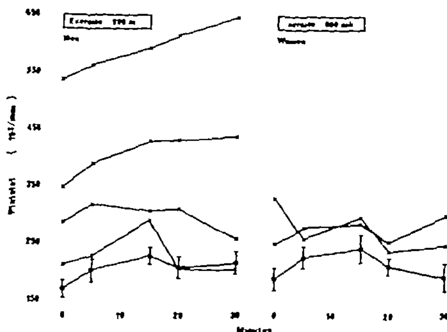


Fig. 1 Effect of propranolol on the rise in the platelet count induced by exercise. Platelet count before and after 15 minutes exercise shown. ○—○ Men (900 mkp) —● women (600 mkp)

work load of 900 mkp and 600 mkp respectively for 15 min. The individual platelet counts are illustrated in figure 2 with the mean values obtained for 6 normal men and women undertaking the same exercise load. The changes in the platelet count induced by exercise in asplenic subjects were variable, but some showed a well-marked transient thrombocytosis. Two asplenic subjects with initially elevated platelet counts had a different pattern of response to exercise with a continued rise in the platelet count after completion of the period of exercise.

Discussion

Studies on the response of the platelet count to exercise have produced differing conclusions. It seems likely that the discrepancies result partly from variations in the severity and duration of the exercise procedure. Most investigators have found that the platelet count at the completion of a prolonged period of physical activity is



ianges in the platelet count induced by exercise in normal and asplenic subjects.
● l subjects (means ± SE) ×— asplenic subjects.

ıanged from the pre-exercise level [6, 9] although Isaacs and ᴐᴏʀᴅᴏɴ [5] judging only on the appearances of blood films, con uded that *the platelet count at the end of a marathon race was* onsiderably elevated. In contrast, most workers have found that short periods of brisk exercise induce a transient thrombocytosis [3 10 11] Using a bicycle ergometer a convenient means of regulating the amount of exercise performed, our results suggest that a critical amount of exercise is required to produce a detectable rise in th platelet count and that this differs between men and women.

The rapidity of the rise in the platelet count during brisk exercise indicates that the thrombocytosis must result from the release of formed platelets into the peripheral blood rather than from increased platelet production

It is well-established that pharmacological doses of adrenaline cause a rapid rise in the level of the circulating platelets. This rise is prevented by prior administration of β-adrenergic blocking agents [7]

and it does not occur in asplenic persons [1]. The absence of a blocking effect of propranolol on the rise in platelets during exercise, and the rise in asplenic subjects, indicate that exercise-induced thrombocytosis is not mediated solely through stimulation of β-receptors by adrenaline and that the spleen is not the major source of the additional platelets appearing in the peripheral blood. BIERMAN *et al.* [2] concluded from their study that the source of increased platelets after intravenous adrenaline was the pulmonary circulation; thus is a possible source of the increased platelets during exercise.

Although the spleen does not appear to be the source of increased platelets during exercise our results suggest that the spleen may have a modifying role in the response to exercise: in its absence 2 subjects had a continuing rise in the platelet count after completion of the exercise. It is notable that these were the 2 subjects with the highest initial platelet counts.

Acknowledgement. We are grateful to the volunteers for their co-operation, to Miss MARY ALLARDYCE, AIMLT for technical assistance and to Dr GILLIAN FOWLER for statistical advice.

Summary

Short periods of brisk exercise induced a rise in the platelet count of normal men and women. This thrombocytosis was not prevented by the prior administration of the β-adrenergic blocking agent propranolol and occurred also in most asplenic subjects tested.

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Authors' address: Dr A. A. Dawson and Dr D. Ogston, Department of Medicine, University of Aberdeen, Foresterhill, *Aberdeen* (Scotland)

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Medical Service (Director D. L. DEUTSCH) USA General Hospital, Frankfurt/M

Severe Megaloblastic Anemia due to Nutritional Vitamin B_{12} Deficiency

R. B LEDBETTER, Jr and E. DEL POZO

In Western societies, the most common cause of vitamin B_{12} deficiency is pernicious anemia. Various clinical conditions may predispose to this deficiency It is stated that the occurrence of significant B_{12} deficiency as a consequence solely of nutritional lack is rare [1 2, 3] This paper documents such a case and reviews the literature on the subject.

Case Report

E.S. (97th Gen H No. 255557) 19 years old white female, was admitted on March 28, 1967 with complaints of fever nausea, excessive fatigue, and shortness of breathing. The fatigue and progressive dyspnea had begun pproximately 2 months before admission. Fever began approximately 24 h prior to admission. Nausea had been noted for 3–4 weeks, with occasional vomiting. The patient denied menorrhagia, hematemesis, melena, diarrhea, or weight loss. She was employed as secretary and appeared to be bly carrying on her work until the day of admission. One of her associates had remarked to the patient just few days before that she looked ery pale. The patient had lived in the United States and Germany and had not travelled to tropical parts of the world. Her only significant past illness was bout of infectious hepatitis in May 1966 requiring 3-week hospitalization and with no apparent residual. Drug intake was limited to birth control pills which she had taken for several months prior to admission. Significantly the patient had been lacto-vegetarian for as long as she could remember

Physical examination revealed ery pale white female, alert and oriented and otherwise well nourished. No jaundice or cyanosis was visible. Temperature 38.6° C, pulse 128, blood pressure 110/70 mm Hg There was no lymphadenopathy or petechia, but on funduscopy an area of hemorrhage was visualized on the temporal side of the left eye.

Laboratory studies. Initial hematocrit 10%, hemoglobin 4 g%, erythrocytes 1 million. Peripheral blood morphology 2 nucleated red cells/100 white cells marked anisocytosis and poikilocytosis, macrocytosis, polychromatophilia and basophilic stippling. Platelets definitely decreased on smear Differential count of 500 leucocytes segmented neutrophils 65.8%, bands 1.8%, metamyelocytes 1.2%, myelocytes 0.2%, promyelocytes 0.4%, lymphocytes 26.4%, eosinophils 0.4%, mononuclears 4.0%. Some of the mononuclears appeared to

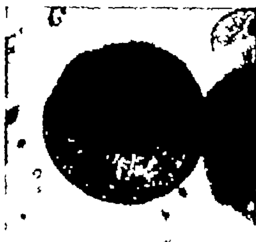


Fig. 1. Megaloblast in bone marrow smear (× 500, photo × 4.25).

have more lobate nucleus than usual. The segmented neutrophils occasionally showed hypersegmentation and slight toxic granulation.

Bone marrow on March, 29: marked megaloblastic erythropoiesis (fig. 1), giant myelocytes, metamyelocytes and bands. Megakaryocytes were decreased and somewhat hyper-lobulated. Iron stain revealed adequate particulate iron. M:E = 1.05:1. Hemoglobin electrophoresis A/A with 2.2% fetal. Coombs test negative. Serum Fe 61 μg%, total iron binding capacity 312 μg%, transferrin saturation 20%. Serum folic acid 10 ng/ml (*Lactobacillus casei* method, normal: 10–20 [4]).

SCHILLING test performed on the 5th hospital day normal with 21.5% of 0.47 μc ^{57}Co-B_{12} excreted in urine in 24 h. D-xylose absorption test [5] normal with 12.4% excretion in urine in 5 h. Normal stomach acidity. Normal upper gastrointestinal and small bowel X-ray study. Serum carotene 70 μg%.

Urine analysis, blood urea, serum protein and electrophoresis, SGOT, total bilirubin, all within normal limits. Blood and throat cultures, stool for ova, parasites and occult blood, all negative. Normal radiographs of chest, skull and femurs.

Hospital course. Initially the patient was given 500 ml of packed red cells slowly and started on prednisone 40 mg daily because of her apparent critical condition. Prior to the bone marrow findings, other diagnoses were strongly entertained such as aleukemic leukemia, pancytopenia, myelofibrosis or myelophthisis of various types. After the bone marrow evaluation consistent with severe megaloblastic anemia, the patient was questioned more specifically regarding dietary history and found to have a strong aversion of long standing to meat products. Prednisone was discontinued as soon as the basic problem was apparent. Parenteral B_{12} was administered 200 μg every 48 h followed by SCHILLING test (1,000 μg), totaling to 2.2 mg B_{12} during the first 2 weeks. A definite hematologic response began about the 5th day and by day 10, the hematocrit, hemoglobin and red cell count had risen to 30%, 10 g% and 3 million respectively. No further B_{12} was given. The hematocrit, hemoglobin and red cell count continued to improve and by day 30 were 40%, 13g% and 5 million. The leucocytes remained low until second month and then returned to normal.

The patient continued to refuse meat products and could not be persuaded to change her abnormal eating habits. Over the next 2 months the patient was asymptomatic and the blood

counts remained stable. However the blood smears changed to macrocytic hypochromic pattern and serum iron confirmed the development of iron deficiency Although iron stains of the initial bone marrow revealed adequate iron stores, the subsequent marked erythropoietic response to B_{12} produced an iron deficiency Apparently the iron reserves were borderline to start with, but appeared normal due to decreased F utilization before therapy

Discussion

A careful review of the literature reveals that severe anemia due to isolated nutritional vitamin B deficiency is very unusual.

A survey of 113 cases of nutritional megaloblastic anemia in India revealed that 54.9% had low serum folate, 5.3% had low serum folate and B_{12}, and 7% had deficiency of vitamin B_{12} alone [6]. This study also ttempted to correlate the serum folate and B_{12} with the hematologic response to therapeutic trials of folic acid and B_{12} parenterally No correlation could be made. The conclusions which can be drawn from this study are that the major cause of megaloblastic anemia in India is folic acid deficiency and that nutritional B_{12} deficiency is more common than in western societies. This is supported by other studies [7 8].

A survey of megaloblastic anemia in the South African Bantu revealed 34 cases associated with pregnancy 16 cases in malnourished infants suffering from marasmus or Kwashiorkor and one case in an adult male vegan [9] The latter patient had free gastric acid after histamine and his hemoglobin rose slowly on normal diet. The other cases responded to therapeutic doses of either B_{12} or folic acid given parenterally I this study no absorption tests, X-rays, or stool examinations for ova and parasites were performed. Therefore, many factors which could hav participated etiologically in the megaloblastic anemia were not excluded.

Moore *et al.* [10] studied series of 23 patients with nutritional macrocytic anemia and evidence of multiple B vitamin deficiencies during 1940–1943 period. Most of these were older than 50 years, and males (widowers or bachelors) who cooked for themselves and therefore ate very poor diet particularly low in animal protein. All but 2 patients had experienced persistent diarrhea for months before admission. Many had evidence of cheilosis, peripheral neuritis, or pellagrous dermatitis. Sternal bone marrows were identical to those of pernicious anemia. Gastric analysis revealed normal acidity in 14, hypochlor hydria in 2, and achlorhydria in 9.

Since these cases occurred before itamin B_{12} or folic acid were isolated and purified, and there were clinical signs of multiple B complex deficiencies in many and evidence of malabsorption in those tested, it cannot be stated clearly whether or not any of these patients was suffering from isolated nutritional B_{12} deficiency Several other case reports in the older literature, prior to the discovery of B_{12} and folic cid, may represent true nutritional B_{12} deficiency but because of possible contributing factors, 'shotgun therapy' and unavailability of specific B_{12} absorption tests or serum levels, this cannot be established with accuracy [11 12, 13 14 15].

In series of 333 cases of megaloblastic anemias studied with serum B_{12} and folate levels by Varadi *et al.* [16] none was due solely to nutritional B_{12} deficiency Fi cases were due to combined dietary deficiency of B_{12} and folate. I addition, there were 161 cases of folate deficiency associated with pregnancy

Woods *et al.* [17] evaluated group of 149 British egans. The MCV of red cells was increased above normal, with the occasional finding of mild anemia. The average serum

B_{12} in British egans was approximately / that of normal Briton. The hematologic changes were disproportionately slight.

SMITH [18] evaluated group of 12 vegans ranging in age from 12 to 71 years and duration of eganism from 5–30 years. None had macrocytic anemia. Perhaps high intake of folic acid in these cases partially compensated for the hematologic deficiency of B_{12}. Interestingly two of these developed combined system disease.

SCHLOESSER [19] reported case of megaloblastic anemia in a young Hindu male vegan, student at the University of Minnesota. An initial SCHILLING test, even with added intrinsic factor was abnormal. Vitamin A and glucose were malabsorbed also. The megaloblastic anemia responded to large doses of parenteral B_{12} and, 15 months later the SCHILLING test had reverted to normal. However the vitamin A and glucose malabsorption did not improve. Tests for fat and folic acid bsorption were normal, but were made more than year after the anemia was corrected. Judging from the bsorption studies, it is probable that this patient had tropical sprue, in addition to nutritional B_{12} deficiency and this combination led to his megaloblastic anemia.

HARRISON [20] presented case of megaloblastic anemia in an elderly female who had been taking diet of bread, margarine, tea and potatoes, plus 200 ml of milk daily for 11 years. In spite of a gastroenterostomy and achlorhydria, she had normal absorption of isotopic B_{12}. Interestingly the patient had consumed Glauber' salt daily for many years with a resultant 5–6 watery stools per day and this may hav produced malabsorptive state Presumably this cathartic was discontinued prior to the radioacti B_{12} absorption test. No folate studies were done, although almost certainly the bove diet was deficient in folate as well. Treatment with parenteral B_{12} 200 μg every 3 weeks resulted in hematologic improvement, however response to this dose of B_{12} is not of itself conclusive of pure B_{12} deficiency

POLLYCOVE [21] reported case of 'pernicious anemia due to dietary B_{12} deficiency in *vegan of 8 years standing. Absorption of B_{12} was normal, however this was not tested until* 3 weeks after therapy with parenteral B_{12} and folic acid was begun. In this regard, it is of interest that intestinal bsorption of B_{12} has been shown to improv following treatment with B_{12} parenterally in various malabsorption states [22, 23]. Furthermore, although this patient undoubtedly had dietary B_{12} deficiency coexistent folate deficiency was not excluded.

BOCHNER [24] reported case of macrocytic anemia in 40-year-old white female whose diet contained no meat or poultry however contained fish once weekly an occasional egg or helping of cheese, and butter Gastric analysis revealed free acid. The initial blood studies showed red cell count of 3.8 million, Hb 11 g%, MCV 94 μm³ Sternal marrow showed changes interpreted as typical of early megaloblastic anemia. A SCHILLING test showed 7.8% excretion after treatment with oral itamin B_{12} had shown slow response. This case reacted suboptimally to super-physiologic doses of B_{12} given orally and even to large parenteral dose given with the SCHILLING test. After repeated parenteral doses of B_{12} over several months, the red cell count and hemoglobin responded to near normal values. An isolated nutritional B_{12} deficiency in this case is questionable in view of the *suboptimal* response to B_{12}. A combination deficiency of folate and B_{12} would be suspected from the *clinical course. A serum folate level might have resolved this question*, but unfortunately was not obtained.

HINES [25] reported case of megaloblastic anemia, in male egan of 13-years standing. A B_{12} bsorption study was normal. Serum B_{12} was low and folate normal. The patient had been habitual blood donor giving one pin of blood every 3 months for the preceding 3–4 years. Furthermore, he had hemorrhoids which had bled small amounts quite frequently The important factor in this case was chronic blood loss, which produced an increased demand for B_{12} in person with borderline reserves. This case offers an interesting similarity to ours, as the latter had been menstruating for 4–5 years.

CONNOR [26] described 75-year-old female vegan of 15 years duration, with megaloblastic anemia, histamine fast achlorhydria and biopsy proven trophic gastritis. The patient was found to bsorb CoB_{12} normally and to have normal serum folate and low serum B_{12}. A good clinical response occurred after parenteral B_{12} therapy. This case probably represents bona fide nutritional megaloblastic anemia due to B_{12} deficiency; however superimposed malabsorption of B_{12} due to atrophic gastritis which later reversed on treatment, cannot be excluded.

A case of megaloblastic anemia in vegan was reported by WREAWER [7]. The patient was 64-year-old female egan for 48 years, who was found to have low serum B_{12}, normal B_{12} bsorption, and had good clinical response to 1 µg of B_{12} orally per day.

Lastly ery special form of utritional B_{12} deficiency should be mentioned. This is known as megaloblastic anemia of infancy and usually occurs in breast fed infants during the first year *post partum*, and has been reported from Southern Europe particularly Italy [29 30] and from India [31]. I several cases reported from India, the mothers were found to have megaloblastic bone marrow low B_{12} in serum and breast milk, and normal SCHILLING tests [31]. Dietary history revealed that the mothers were egans or lactovegetarians. LAMPKIN [32] reported case of this entity from Los Angeles in which mother was subsequently found to have pernicious anemia. The mother' breast milk was found to be ery low in B_{12} compared to milk from normal mothers. The old adage that 'mother milk is best obviously is not true under these circumstances.

The patient described in our report had consumed no meat and only occasionally had taken milk or milk products for about 13 years, because of a personal distaste for animal flesh. She had remained well enough to carry on a sedentary job until a few days before admission, although she had felt progressively ill over a 2 month period. Her appearance on admission was that of an acutely ill, pale, toxic young female in good general nutrition. The initial impression was that she had either acute terminal leukemia or pancytopenia. Not until the bone marrow results were available did the examiner question her regarding dietary habits. This suspicion was aroused because of the rarity of pernicious anemia at this age, or folate deficiency in an otherwise well nourished young non-pregnant female. The dietary history coupled with normal gastric acidity and B_{12} absorption (on the 5th hospital day) normal serum folate, and complete response to parenteral vitamin B_{12}, made the diagnosis of isolated nutritional B_{12} deficiency almost certain. The borderline low D-xylose absorption and serum carotene were difficult to explain, especially in view of the normal upper gastrointestinal and small bowel series, and normal B_{12} absorption. Perhaps there was some degree of malabsorption secondary to the effect of chronic B_{12} deficiency on the epithelium of the small bowel [28] which did not affect B_{12} or folate absorption.

The present case along with that of HINES [25] CONNOR [26] and

WINTRAWER [27] represent those megaloblastic anemias in adults which most clearly are due to isolated nutritional deficiency of B_{12}.

Summary

A case is presented of severe megaloblastic anemia due to a nutritional deficiency of vitamin B_{12} in young female lactovegetarian otherwise in good health and nutrition, and an attempt is made to review the literature pertaining to this subject.

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Authors' addresses: Dr. Rudi B. Lindemann, Physical Evaluation Board, US Army Hospital, *Ft. Gordon, Ga.* (USA); Dr. Emilio del Pozo, Obere Mattstrasse 88, *4133 Pratteln* (Switzerland)

H. J. Bendixen (editor) **Leukemia in Animals and Man.** 3rd International Symposium for Comparative Leukemia Research, Paris 1967. Bibliotheca Haematologica No. 30. Karger Basel/New York 1968. XII + 348 p., 89 fig., 1 cpl., 56 tab. Price sFr./DM 79.– / US $ 18.95/£ 6.8 s.

Since 1963 the World Committee for Comparative Leukemia Research promotes the co-operation of leukemia studies in man and animals by organizing symposia every second year. The proceedings of the 3rd symposium held in Paris 1967 are now being published. More than 80 contributions cover the large spectrum of basic research, pathogenesis, incidence and geographical distribution of leukemia in human and veterinary medicine. The reported main lectures are: Determinants of Viral Carcinogenesis (J. L. Melnick); Interactions of RNA Oncogenic Viruses and Cells (P. Vigier); Viral Research on Bovine Leukosis (R. M. Dutcher); The Epidemiology of Bovine Leukosis (R. R. Marshak and D. A. Abt); Advances in Feline Leukemia (O. Jarrett *et al.*) and Recent Studies on Leukemia and Solid Tumors in Mice and Man (L. Dmochowski). Unfortunately only a short abstract is given of F. J. Rauscher's main lecture 'Progress in the Viral Research of Human Leukemia'. In a large number of additional communications a variety of recent results in leukemia research are presented, most of them dealing with viral etiology, immunology and electron microscopic observations. The book provides an excellent review of the actual basic knowledge in the field of leukemia and can be recommended to every hematologist. The subscribers to *Acta Haematologica* and *Vox Sanguinis* receive the volume at a reduced price.

H. R. Marti, *Aarau*

R. Gross **Medizinische Diagnostik. Grundlagen und Praxis.** Heidelberger Taschenbücher Band 48. Springer Berlin 1969. 218 S., 12 Abb., 14 Tab. Preis DM 9.80, $ 2.45.

Die vorliegende Monographie, die weit mehr als den Namen eines Taschenbuches verdient, habe ich, bevor das Besprechungsexemplar eingetroffen war, in einem Zug und mit grossem Genuss und Gewinn durchgelesen. Die wohl abgewogenen 6 Kapitel mit reichlich Zitaten und Literaturangaben zeigen dem Praktiker, aber auch dem klinisch tätigen Arzt die verschiedenen Aspekte der Diagnostik, auch ihre Grenzen, Fehlerquellen und Abstufungen, die breite Fächerung und Bedeutung der Laboruntersuchungen und die mathematisch-maschinellen Methoden in ihrer zunehmenden Wichtigkeit. Beherzigenswerte Ausführungen liegen über die Labor-Gläubigkeit vor, wobei in der Praxis auffällt, wie kleine Abweichungen von der Norm oft überbewertet werden und nicht selten die Normalwerte schlechter bekannt sind als die gängigen Börsenkurse. Alles in allem eine ausgezeichnete Zusammenstellung über ein Gebiet, das zur ärztlichen Klinik gehört und im Zeitalter der aufkommenden Computer immer wieder zu überdenken ist. Gerade auch im Hinblick auf den zitierten Ausspruch von M. McLean: *«Ein Spezialist ist ein Mensch, der auch nicht den kleinsten Fehler macht, während er auf den grossen Trugschluss zusteuert.»*

F. Wuhrmann, *Winterthur*

H. U. Zollinger **Pathologische Anatomie.** Thieme, Stuttgart 1968. Vol. 1: Allgemeine Pathologie. XII + 335 p., 136 meist zweifarbige Abbildungen in 197 Einzeldarstellungen. Flexibles Taschenbuch. Preis DM 9.80. Vol. 2: Spezielle Pathologie. XVI + 560 p., 375 meist zweifarbige Abbildungen in 555 Einzeldarstellungen. Flexibles Taschenbuch. Preis DM 14.80.

In verdankenswerter Weise hat es der Verlag Thieme unternommen, seine bekannte und gut eingeführte Serie der Taschenbücher auf ein grosses Gebiet wie die pathologische Ana-

tomie auszudehnen. Dass dieser Versuch gelungen ist, ist in erster Linie darauf zurückzuführen, dass dafür ein im Unterricht so erfahrener Lehrer wie Prof. Zollinger als Autor gewonnen werden konnte. Wenn diese Bücher eine Bausteinsammlung bieten sollen, wie einleitend gesagt wird, so handelt es sich um eine sehr vollständige Sammlung, die in zwar knapper aber erschöpfender Weise die wesentlichen Daten vermittelt, die der Student benötigt. Aller überflüssige Ballast ist weggelassen, dafür werden die Grundlagen um so solider und klarer herausgearbeitet. Es darf sogar erwartet werden, dass ein Student mit diesen Kompendien, ergänzt und vertieft durch eine umfassende Vorlesung, auszukommen vermag. Eine klare und logische Gliederung des Stoffes und ein ausführliches Sachverzeichnis erleichtern das Studium und erlauben eine rasche Orientierung über jeden Fragenkomplex. Besonders hervorzuheben und in ihrer Art beispielhaft sind die zahlreichen skizzenhaften Abbildungen, die besser als jede Beschreibung die wesentlichen Befunde wiedergeben und über die reine Morphologie hinaus auch pathophysiologische Zusammenhänge zur Darstellung bringen.

Der erste Band vermittelt die Grundlagen der allgemeinen Pathologie (Missbildungen, regressive und progressive Veränderungen, Störungen von Atmung und Kreislauf, Entzündung, Tumoren, exogene Noxen). Im zweiten Band werden diese Grundlagen auf die einzelnen Organsysteme bezogen, wobei gerade hier die Ausblicke auf Pathophysiologie und Klinik besonders wertvoll und anregend sind. Die hämatologischen Erkrankungen, sonst ein Stiefkind der pathologisch-anatomischen Lehrbücher, finden gebührende Würdigung, wobei sogar die hämorrhagischen Diathesen in ihrer Symptomatologie und Pathogenese dargestellt sind.

Autor und Verlag haben mit diesen beiden Bänden bewiesen, dass dem Studenten auch auf einem grossen Gebiet der Medizin mit dem Taschenbuch ein wertvolles Lehrmittel in die Hand gegeben werden kann. Darüber hinaus ist die Lektüre auch für den Kliniker und den praktisch tätigen Arzt, der über ein Gebiet eine rasche Orientierung sucht, nicht nur nutzbringend, sondern in mancher Hinsicht anregend.

H. Lüdin, *Basel*

E. Deutsch, E. Gerlach and K. Moser: **Metabolism and Membrane Permeability of Erythrocytes and Thrombocytes.** Thieme, Stuttgart 1969. XVI + 480 p., 407 fig., 101 tab. Preis DM 110.

Biologische Membranen sind in den letzten Jahren dank der Entwicklung neuer physikochemischer Methoden auf immer grösseres Interesse gestossen. Fragen des allgemeinen Zellstoffwechsels sind aufs engste damit verknüpft. Die Zellen des Blutes eignen sich ganz besonders für solche Untersuchungen. Da das Schrifttum bald nicht mehr zu überblicken ist, war es ein glücklicher Gedanke, die verschiedenen am Membran- und Zellstoffwechsel interessierten Arbeitsgruppen zu einem gemeinsamen Gespräch in Wien zusammenzurufen. Gegen zweihundert Teilnehmer haben diesem Ruf Folge geleistet. Der vorliegende Band umfasst sämtliche (nahezu 100) vorgetragenen Referate, zum Teil in etwas gekürzter Form. Die ebenfalls wiedergegebenen Diskussionsvoten sind redaktionell geschickt bearbeitet worden.

Eine erste Gruppe von 23 Arbeiten befasst sich mit dem Stoffwechsel normaler Erythrocyten. Die folgenden 9 Artikel sind dem Stoffwechsel pathologischer Erythrocyten gewidmet, wobei auch die Bedeutung der Milz kurz gestreift wird. Eine weitere Gruppe beschäftigt sich mit den enzymatischen Analysenmethoden. Ein besonders grosser Abschnitt ist dem Stoffwechsel und der Funktion der Thrombocyten gewidmet. Die drei letzten Kapitel befassen sich mit den Membranen im engern Sinn (Thrombocytenmembran 11 Arbeiten, Erythrocytenmembran 12 Arbeiten, Permeabilität der Erythrocytenmembran 15 Arbeiten).

Die Zuteilung der Artikel zu den einzelnen Kapiteln scheint nicht immer zwingend; das übersichtliche Inhaltsverzeichnis und ein Sachregister erleichtern aber das Auffinden

H. J. Bendixen (editor): **Leukemia in Animals and Man.** 3rd International Symposium for Comparative Leukemia Research, Paris 1967. Bibliotheca Haematologica No. 30. Karger Basel/New York 1968. XII + 348 p., 89 fig., 1 cpl., 56 tab. Price: sF ./DM 79.–/ US $ 18.95/158 s.

Since 1963 the "World Committee for Comparative Leukemia Research" promotes the co-operation of leukemia studies in man and animals by organizing symposia every second year. The proceedings of the 3rd symposium held in Paris 1967 are now being published. More than 80 contributions cover the large spectrum of basic research, pathogenesis, incidence and geographical distribution of leukemia in human and veterinary medicine. The reported main lectures are: Determinants of Viral Carcinogenesis (J. L. Melnick). Interactions of RNA Oncogenic Viruses and Cells (P. Vigier). Viral Research on Bovine Leukosis (R. M. Dutcher). The Epidemiology of Bovine Leukosis (R. R. Marshak and D. A. Abt). Advances in Feline Leukemia (O. Jarrett et al.) and Recent Studies on Leukemia and Solid Tumors in Mice and Man (L. Dmochowski). Unfortunately only a short abstract is given of F. J. Rauscher's main lecture "Progress in the Viral Research of Human Leukemia". A large number of additional communications with a variety of recent results in leukemia research are presented, most of them dealing with viral etiology, immunology and electron microscopic observations. The book provides an excellent review of the actual basic knowledge in the field of leukemia and can be recommended to every hematologist. The subscribers to "Acta Haematologica" and "Vox Sanguinis" receive the volume at a reduced price.

H. R. Marti

R. Gross: **Medizinische Diagnostik. Grundlagen und Praxis.** Heidelberger Taschenbücher Band 48. Springer, Berlin 1969. 218 S., 12 Abb., 14 Tab. Preis DM 9.80; $ 2.45.

Die vorliegende Monographie, die weit mehr als den Namen eines Taschenbuches verdient, habe ich, bevor das Besprechungsexemplar eingetroffen war, in einem Zug und mit grossem Genuss und Gewinn durchgelesen. Die wohl abgewogenen 6 Kapitel mit reichlich Zitaten und Literaturangaben zeigen dem Praktiker, aber auch dem klinisch tätigen Arzt die verschiedenen Aspekte der Diagnostik, auch ihre Grenzen, Fehlerquellen und Abstufungen, die breite Fächerung und Bedeutung der Laboruntersuchungen und die mathematisch-maschinellen Methoden in ihrer zunehmenden Wichtigkeit. Beherzigenswerte Ausführungen liegen über die Labor-Gläubigkeit vor, wobei in der Praxis auffällt, wie kleine Abweichungen von der Norm oft überbewertet werden und nicht selten die Normalwerte schlechter bekannt sind als die gängigen Börsenkurse. Alles in allem eine ausgezeichnete Zusammenstellung über ein Gebiet, das zur ärztlichen Klinik gehört und im Zeitalter der aufkommenden Computer immer wieder zu überdenken ist. Gerade auch im Hinblick auf den zitierten Ausspruch von M. McLuhan: „Ein Spezialist ist ein Mensch, der auch nicht den kleinsten Fehler macht, während er auf den grossen Trugschluss zusteuert."

F. Wuhrmann, Winterthur

H. U. Zollinger: **Pathologische Anatomie.** Thieme, Stuttgart 1968. Vol. 1: Allgemeine Pathologie. XII + 335 p., 136 meist zweifarbige Abbildungen in 192 Einzeldarstellungen. Flexibles Taschenbuch. Preis DM 9.80. Vol. 2: Spezielle Pathologie. XVI + 560 p., 373 meist zweifarbige Abbildungen in 533 Einzeldarstellungen. Flexibles Taschenbuch. Preis DM 14.80.

In dankenswerter Weise hat es der Verlag Thieme unternommen, seine bekannte und gut eingeführte Serie der Taschenbücher auf ein grosses Gebiet wie die pathologische Ana-



Ludwig Heilmeyer

Prof. LUDWIG HEILMEYER, who died unexpectedly on September 6 at the age of 70, was particularly interested in haematology. Already many years ago, when he was Associate Professor of Medicine at the Medizinische Universitätsklinik in Jena, he published his studies on Iron therapy, Serum iron and Iron Deficiency.

Later, when he held the chair of Internal Medicine at the University of Freiburg i. Br., he was the author of the outstanding Atlas der klinischen Hämatologie (with BEGEMANN) and edited the comprehensive Handbuch der gesamten Hämatologie. Since ACTA HAEMATOLOGICA were founded (1948) HEILMEYER was very active as one of our Contributing Editors. A number of his important papers appeared in our journal.

The International Society of Haematology had elected HEILMEYER President for the meeting which will take place in München 1970.

His untimely death is deeply felt.

The Editors-in-Chief and the Publisher

Photo: Willy Pragher, Freiburg i. Br.

Acta haemat. 42: 258–268 (1969)

Department of Anatomy, State University of New York at Buffalo, Buffalo, N.Y.

Electron Microscopic Study of the Granules in Guinea Pig Bone Marrow Basophils[1]

BEATRICE S. T. CHAN[2]

It is generally agreed that the three principal types of granulocytes develop by a common pattern of maturation through the stages of myeloblast, progranulocyte, myelocyte, metamyelocyte, band cell and mature cell. During development the three cell lines – neutrophil or heterophil, eosinophil and basophil – can be identified as early as in the progranulocyte stage by the appearance of their specific granules. It would be, therefore, interesting to know how these specific granules develop from a common ancestral myeloblast. The development and maturation of the granulocytes and their granules had been studied previously by PEASE [8, 9], WINQVIST [17], BAINTON and FARQUHAR [1], TAKEOKA *et al.* [11] and WETZEL *et al.* [14], and of the mast cells and their granules by HIBBS *et al.* [6], THIERY [12], COMBS *et al.* [4] and COMBS [3]. The exact mode of formation of basophil granules, however, has not been elucidated. This paper is to report electron microscopic observations on the granules of developing basophils in the guinea pig bone marrow.

Material and Methods

Thirteen male guinea pigs of Dunkin-Hartley strain, weighing 400–600 g, were used. Previous investigations [2, 15] showed that the production of basophils in the bone marrow was increased after administration of horse serum. In order to have a greater number of basophils for the study, a single i.p. injection of 0.5 ml of horse serum was given to each

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[2] On leave from the University of Hong Kong, under a Dr. Henry C. and Bertha H. Buswell Fellowship grant of the State University of New York at Buffalo.

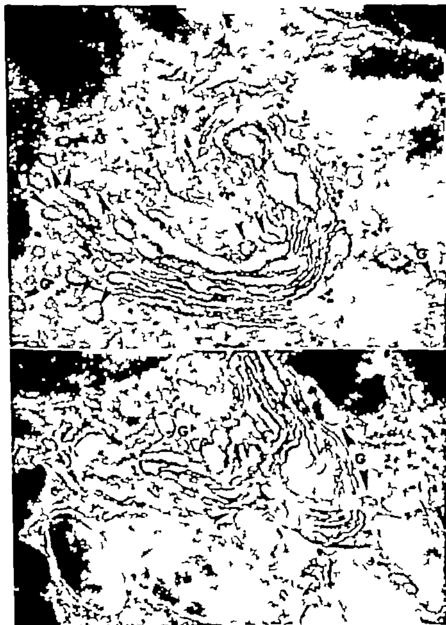


Fig 1 Golgi complex of basophil myelocyte. Budding off of granule precursors (arrows) from the ends, and tw from the conca side of Golgi cisternae. Some (G) are scattering in the vicinity of Golgi complex (10,600) *b* A finely granular acuole is pinching off from the convex side of Golgi cistern (arrow). Small, young granules (G are seen close to the Golgi complex 10 600

animal 7 days before it was killed. Perfusion-fixation with warm 0.1 M phosphate-buffer solution, pH 7.4 followed by cold 4 glutaraldehyde in the same buffer was performed through the abdominal aorta of the animal under chloral hydrate anesthesia (1 ml of 3.5 aqueous chloral hydrate per 100 g body weight) Both femora were removed immediately after perfusion and the pencils of bone marrow were immersed quickly into fresh cold 4% glutaraldehyde. The bone marrow was then cut into small pieces (0.1–0.2 mm thick) with sharp razor blade, and the chosen pieces were reimmersed in fresh 4% glutaraldehyde for ½–1 h. They were then postfixed in phosphat -buffered 1 % osmium tetroxide for 1½ h at 4 C [7] The tissue specimens were dehydrated with graded ethanol, and embedded in Maraglas mixture [10]

Ultrathin sections were cut on Sorvall Porter Blum MT 2 ultramicrotome with glass knives. They were stained with lead citrate [13] prior to examination with JEM-6C electron microscope.

Results

The majority of basophils observed in the bone marrow sections were myelocytes and metamyelocytes, which were characterized by their relatively large and oval-shaped granules and their eccentric nucleus. The basophils could usually be distinguished from the eosinophils because the latter had smaller granules which were often elliptical in shape with one (occasionally two) rectangular profiled crystalline structure

The Golgi complex was very prominent in the basophil myelocytes and metamyelocytes. Profiles of centriole were occasionally observed in close relationship to the Golgi complex The cytoplasm of myelocytes and metamyelocytes contained granules of different stages, numerous ribosomes and abundant rough-surfaced endoplasmic reticulum which appeared slightly dilated with a finely particulate material of medium opacity in the cisternae Mitochondria were large and moderately dense

Formation of basophil granules During electron microscopic examination of the bone marrow sections the Golgi complex was prominently in view in the developing basophils. Its flattened cisternae became dilated and sacculated with a lower electron-opaque material than the surrounding cytoplasmic matrix. As the saccules of the cisternae were well formed, they seemed to have pinched off one after another from the ends of the cisternae (fig 1a) and from both sides of the sacculations along the cisternae to form vacuole like granules (fig 1b) Some of them were still connected to the cisternae and had not yet completel separated from them These vacuole-like granules of various sizes were seen scattering in the vicinity of the Golgi complex between the



Fig. 2. Golgi complex of basophil myelocyte showing the distribution of the immature granules (G) between cisternae on both the convex and concave surfaces, and around the Golgi complex. Er = endoplasmic reticulum (× 10,600)



Fig. 3. Two basophil granules showing their close relationship with the rough-surfaced endoplasmic reticulum Er. The continuity between the limiting membrane of granule and the cistern of endoplasmic reticulum is shown by arrow. 10,600

cisternae on the convex and concave faces and around the sides of the Golgi complex (fig 2) The definite granules of early forms were membrane bound and had a finely granular structure of medium density They looked quite similar to the cross-sectioned cisternae of the endoplasmic reticulum They were identifiable by their smooth outline, and denser and more osmiophilic content while the endoplasmic reticulum usually had a rough outline studded with ribosomes, and was relatively lighter in density and less osmiophilic.

Aggregation of small granules to form larger granules might be present in basophils in the process of development but it did not seem to be so evident as in the developing eosinophils The content of some basophil granules appeared coarser than that of the others. As the granules moved away from the Golgi region, they were often seen to have close relationship with the rough-surfaced endoplasmic reticulum the latter either surrounded the granules or contacted their limiting membrane. In some instances, a continuity could be observed between the limiting membrane of the granules and the cisternae of the endoplasmic reticulum (fig 3 arrow) The electron density of the granules seemed to increase with the maturity of the granules. There might be reorganization and condensation of the granule content for the mature granules appeared to be smaller denser and more homogeneous.

All the basophil granules had a well-defined limiting membrane beneath which a low electron-dense area of varying width might be present and appeared like a halo (fig 3)

Internal ultrastructure of basophil granules The majority of basophil granules in myelocytes and metamyelocytes showed a lamellated structure (fig 4a) The lamellation consisted of two-dimensional sets, each having *a series of parallel electron-opaque lamellae of about equal* thickness. These lamellae were spaced regularly apart and the spacings were about the same in thickness as the lamellae The lamellation therefore, appeared as a series of alternate dense and light lines (fig. 4a) *One lamella and its adjacent spacing together measured* 10 nm thick. The lamellae of both sets ran obliquely to the long axis of the granule but in different directions and at an angle of 112.5 degrees between them they interdigitated to produce a regular network (fig 5) In the sections, therefore four possible types of lamellation could be seen The granules might be cut along the plane of either set of the lamellation (fig 4a A) or obliquely to the lamellae (fig 4a, B) the sections might be exactly at the intersections of the two



Fig. 4e Basophil granules. *A* Lamellation cut along the plane of the lamella th series of alternate dense and light lines of equal thickness. *B* Lamellation cut obliquely to the lamellae 25,000 *b* Higher magnification of basophil granule showing honeycomb lamellation (25,000

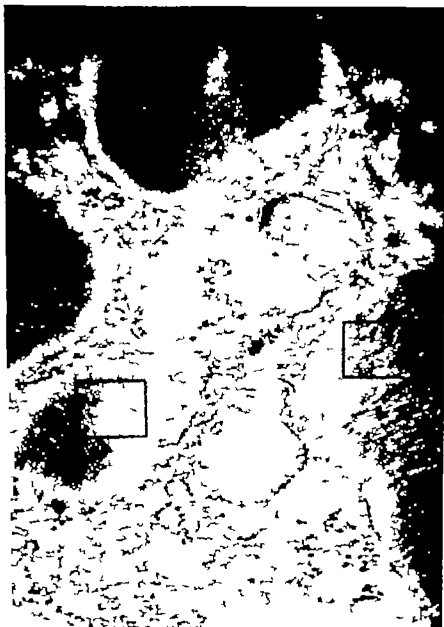


Fig. 5. Basophil granules showing interdigitations of the lamellae with each other (squares) and the dotted appearance of the granule (D) when cut between lamellae of one set and across those of the other (× 25,000)

sets of lamellae (honeycomb pattern in fig. 4b, C) or right across the spacing of one set and the lamellae of the other (fig 5 D)

Discussion

The formation of granules in the basophils of guinea-pig bone marrow observed in this investigation is essentially similar to that in neutrophil and eosinophil series described by BAINTON and FARQUHAR [1] and WETZEL *et al* [14] namely that the granule precursors are produced from Golgi complex. These authors reported that the granule precursors are formed by budding off from the ends of Golgi cisternae My observations show however that the formation can take place at the ends as well as along the sides of the cisternae The granules seen in the vicinity of Golgi complex are of various sizes and of slightly different density This may indicate different stages of the granule development. The granules are closely related to the endoplasmic reticulum through which new material may be continuously added to the granules. Further maturation of the granules may probably take place by reorganization and condensation, because the mature granules are often smaller and denser than the immature ones.

It was noted in the bone marrow sections that the basophil granules are limited by a membrane and that some granules are surrounded by a halo of electron density beneath the limiting membrane. These features may be due possibly to partial separation of the granule from its membrane as a result of fixation and dehydration

Two sets of lamellation are present in the basophil granules. The third-dimensional lamellation which, if present [5] would run longitudinally along the long axis of the granule has not been observed. In any longitudinal sections of the granules, the lamellae are never running straight on the sections from one end to the other Furthermore if the lamellations were three-dimensional at least one set of the lamellae in any possible sections would appear either straight or oblique and the whole granule would never be honeycomb in appearance as seen in figure 4b

During my examination of basophil granules, the formation of eosinophil granules was also observed. Though the eosinophil granule precursors are produced in the same manner as the basophil precursors from the Golgi complex, the content of the former is different from

that of the latter In the eosinophils some highly electron-dense material appears first inside the cisternae of Golgi complex. This dense material then accumulates to form the core of the granule precursors The aggregation of immature granules from the small to the big ones is more evident in eosinophils than in basophils Intimate relationship of the immature granules with the endoplasmic reticulum has also been seen in the eosinophils. Synthesis, reorganization and condensation of the granule constituents may also be suggested for further maturation of eosinophil granules

Acknowledgment. I wish to express my sincere gratitude to Dr OLIVER P JONES and Dr JOSEPH C. LEE for reading my manuscript, for their comment, and for the laboratory facilities available to me during the course of this investigation. I also wish to thank M JOHN WIRTH and Miss A r SORESON for their technical assistance

Summary

The development and maturation of basophil granules were studied electron-microscopically in guinea-pig bone marrow The granules of basophils ppeared t be produced by budding off from the Golgi complex. There might be aggregation of small granules to form larger ones during the process of developmen and the granules migh also increase their constituents and size by continuous contribution of new material from the adjacent rough-surfaced endoplasmic reticulum. The basophil granules ere haracterized by lamellar ultrastructure which is composed of two-dimensional sets, running obliquely to the long axis of the granule and interdigitating ith each other at an angle of 112.5 degrees. Each lamella and its adjacent spacing (one dense and one light line together) measured about 10 nm in thickness.

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Author's address: Dr. BEATRICE S. T. CHAN, University of Hong Kong, Department of Anatomy, L. Shu Fan Building, Sassoon Road, *Hong Kong* (Hong Kong)

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Department of Medicine, University of Cambridge, Cambridge

Periodic Acid-Schiff Reaction in Human Basophilic Leucocytes

D. T. Kauno

Relatively few studies have been made on the PAS reaction and glycogen content of the basophilic leucocytes because of their scarcity in the peripheral blood. Basophils are generally considered to be PAS positive. However, opinions differ regarding the substance responsible for the PAS positivity. Smith [1] and Wislocki *et al.* [2] showed that the PAS positive material represented glycogen and was completely removed by salivary diastase. Astaldi [3] found the granules to be only partially diastase labile. Ackerman [4] found both diastase labile and resistant granules, the latter probably containing heparin monosulfuric ester. There was also disagreement regarding the identity of the basophilic and the PAS positive granules. Smith [1] found the basophilic granules to be PAS negative. Ackerman [4] stated that the diastase resistant PAS positive granules represented the basophilic granules. Dunn and Spicer [5] also suggested that the basophilic granules were PAS positive. In view of these controversies, this study was undertaken to determine the PAS reactivity of the basophilic granules and the diastase lability of the PAS positive granules.

Methods

Air dried peripheral blood smears or buffy coat smears were stained with the Leishman technique. The basophils were identified and photomicrographs were taken. The slides were then stained with the PAS technique according to the method of Hayhoe *et al.* [6]. The same cells were again identified and photographed. Salivary amylase digestion was done before the periodic acid treatment. Specimens for electron microscopy were prepared according to the methods of Anderson [7] and Watanabe [8]. One μm thick Araldite

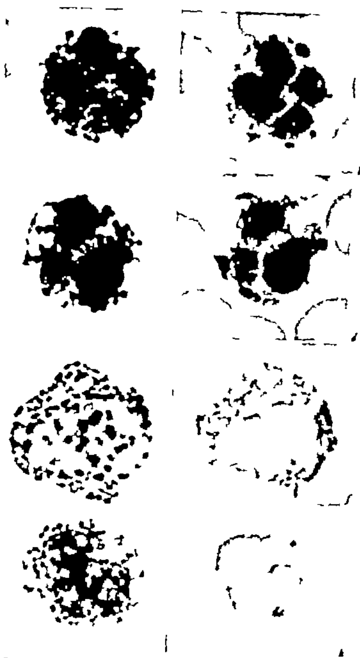


Fig. 1 Basophils, consecutive Leishman (left) and PAS stains (right). *a*, *b*, *c*, *d* from patient with chronic myelocytic leukaemia, *f* from normal man, *g*, *h* from another patient with chronic myelocytic leukaemia.

sections directly adjacent to the ultra-thin sections were mounted on microscopic slides and stained with the PAS technique. Salivary amylase digestion was done prior to fixation with glutaraldehyde.

Results

Consecutive Leishman and PAS stains Photomicrographs of 36 basophils from 3 normal men and 2 patients with chronic myelocytic leukaemia and elevated basophilic counts were compared The discrete basophilic granules were PAS negative The discrete PAS positive granules corresponded with clear spaces in the Leishman stained specimens (fig 1) Some basophils showed no discernible PAS positive granules (fig 2) In a few areas where there were dense aggregates of basophilic granules, faint PAS positivities could be seen which did not correspond in sizes or shapes to the basophilic granules. There was no apparent difference between the normal and leukaemic basophils *except that some basophils of one of the leukaemic patients* showed unusually large blocks of PAS positive material

Salivary amylase digestion. Buffy coat smears from 4 normal men were stained with the PAS technique with and without salivary amylase digestion. In the untreated smears PAS positive basophils were readily *identified No PAS positive cells could be seen in the smears previously* treated with salivary amylase Similarly smears from 3 patients with chronic myelocytic leukaemia and white cell counts of 15 600 (18 basophils) 9 800 (6.6 % basophils) and 83 700 (3 6 basophils) showed no PAS positive basophils after salivary amylase digestion.

Electron microscopy Seven basophils in ultra thin and thick section pairs from one patient with chronic myelocytic leukaemia and elevated basophilic count were compared. Three basophils in ultra thin sections showed conspicuous localized aggregates of glycogen in addition to scattered particles in the cytoplasm. These areas corresponded well with the PAS positivities in the adjacent thick sections of the same cell On the other hand areas which contained aggregates of basophilic granules in the ultra-thin sections showed no PAS positivity in the corresponding areas of the thick sections (fig 3) The glycogen particles in the ultra thin sections were completely removed by salivary amylase digestion

It would appear from these studies that basophilic granules are not identical with the PAS positive granules. Since the PAS positivity

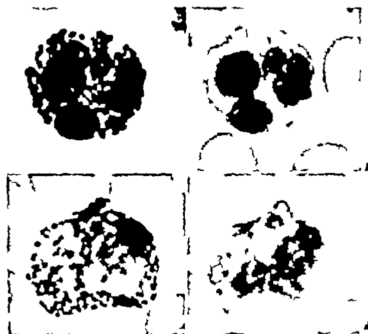


Fig. 2 Basophils, consecutive Leishman (left) and PAS stains (right) *a, b* from patient with chronic myelocytic leukaemia, *d* from normal man.

is completely removed by salivary amylase, it must represent glycogen in the cytoplasm of the cell. The diastase labile particles in the ultra thin sections stained with lead were also identified as glycogen [8 9] The close proximity of these glycogen particles to the basophilic granules as shown previously by several workers [6 7 10] and also in this study may well explain the occasional overlapping of PAS positive material and basophilic granules.

Acknowledgments. The author is indebted to Professor F G J HAYHOE for his helpful suggestions and to Messrs. R. J FLEMANS and M. A. PEACOCK for the microscopic and electron microscopic reproductions.

Summary

By the use of consecutive Romanowsky and PAS stains and the correlation of ultra-thin and thick sections, basophilic granules in human leucocytes were found to be PAS negative. The PAS positive material in the cytoplasm corresponded to the extra-granular glycogen which was removed by salivary amylase



Fig. 3. Adjacent thick and ultra-thin sections of basophils. Ultra-thin section stained with lead citrate (× 12,800) thick section stained with PAS.

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Author address Dr D T KAUNO, Medical Service Veterans Administration Hospital, Iowa City Ia 52240 (USA)

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Department of Internal Medicine, Research Institute for Nuclear Medicine and Biology
Hiroshima University (Director Prof H Uchino) Hiroshima

Ultrastructural Localization of Alkaline Phosphatase in Human Neutrophils

T Nakatsui

Since the histochemical demonstration of alkaline phosphatase in leukocytes was established by Wachstein [1] in 1946 several methods have developed and its clinical significance has been clearly ascertained in some disorders. The ultrastructural localization of alkaline phosphatase was also found in such tissues as kidney intestine and cerebrum [2] Recently fine structural localization of this enzyme in rabbit heterophil leukocytes was described [3 4] but not that in human neutrophil leukocytes.

In the present study the ultrastructural demonstration of alkaline phosphatase in human neutrophil leukocytes was undertaken according to Mayahara s alkaline phosphatase procedures [2] which have recently been developed for tissue section staining

Materials and Methods

Heparinized venous blood obtained from woman was centrifuged for 5 min at 500 rpm and the supernatant was centrifuged again for 5 min 2,000 rpm in cold room. The leukocyte sediment was suspended in saline solution and rinsed three times, and the sediment was added to Mayahara's alkaline phosphatase incubating medium which was prepared just before incubation. Immediately after adding the medium, the specimens were frozen in dry ice and alcohol mixture at –80° C, then thawed at room temperature and allowed to stand for 3 h at room temperature without exchange of the medium. The incubating medium consists of 1 4 ml of 0.3 M tris-HCl buffer pH 8.5 (28 mM in the final concentration) 2.0 ml of 0.1 sodium β-glycerophosphate (20 mM) 2.6 ml of 15 mM magnesium sulfate (3.9 mM) and 4.0 ml of 0.5% alkaline lead citrat solution, pH c. 10.0 (2.0 mM) Sucrose may be added in final concentration of 8%. Final pH is adjusted to pH 9 2 9.4 with 0.1 HCl. After 3 h incubation, the specimens were centrifuged, rinsed with cacodylate buffer fixed



Fig. 1 Mature neutrophil leukocyte from venous blood of female. Some types of granules contain dense reaction product indicative of alkaline phosphatase activity showing reaction in (1) medium-sized granules, (2) large vacuoles or autophagic vacuoles, and (3) small round or oval granules (10,000

in cacodylate buffered 3 % glutaraldehyde and 1 % osmium tetroxide for 1 h at 4 C, dehydrated by routine procedures and embedded in Epon. Thin sections stained with lead acetate were examined in Hitachi HS 7 type electron microscope. As the control, the incubating medium without substrate was used for the specimens in the same procedure.

Results

Reaction product was found in the cytoplasm of mature neutrophil leukocytes as follows (1) medium-sized round granules, some of which contained dense materials (fig 3) (2) membrane of large vacuoles or autophagic vacuoles, some of which fused with reaction positive



Fig 2 Mature neutrophil leukocyte showing reaction positive granules (8,000)

granules (fig 1) and (3) small round or oval granules and sometimes a long tubular duct in the peripheral region of the cytoplasm These are scanty in the central region of the cells (fig 1 2) In the absence of substrate no reaction products were observed.

Discussion

Technical considerations Since the activity of human neutrophil alkaline phosphatase is low it is difficult to demonstrate this enzyme ultrastructurally in good preservation of fine structures. It usually needs 15 h incubation in GOMORI's lead salt method to stain alkaline



Fig. 3. Medium-sized granules which contain reaction product around the dense materials (× 10 000)

phosphatase in human leukocyte smears histochemically whereas in other tissues, such as kidney intestine or cerebrum, it needs only 1 and ½ h incubation

It was found in the preliminary trials that glutaraldehyde and para formaldehyde fixation inactivated the activity of this enzyme. In the present study no fixatives were used before incubation. Due to the phagocytic function of the neutrophilic leukocytes there is a possibility that the neutrophils take in some reaction precipitates which react with serum alkaline phosphatase for this reason the leukocyte suspension in the incubating medium was allowed to undergo immediate freezing and thawing in order to prevent phagocytosis.

Among several methods for ultrastructural demonstration of this enzyme no precipitates were formed in MAYAHARA's new lead-citrate method in high alkaline pH without chelating agent which is known to have an inhibitory effect on this enzyme

Localization of alkaline phosphatase in human neutrophil leukocytes. It was reported that fine structural studies of heterophil leukocytes in rabbit had revealed heterogeneity among their cytoplasmic granules and at least three types of heterophil granules had been distinguished namely (1) large dense primary or azurophil granules, (2) the smaller less dense secondary granules, and (3) tentatively defined tertiary granules small pleomorphic dense granules)

WETZEL [3] demonstrated for the first time alkaline phosphatase in secondary granules of rabbit heterophil myelocytes by electron microscopy which appeared in intermediate heterophils and later predominated in mature cells, and also demonstrated that this

enzyme in Golgi complex coincided with secondary granulocytogenesis. Bainton [4] also showed this enzyme in Golgi cisternae and immature specific granules of rabbit heterophil myelocytes. Horn [6] described alkaline phosphatase in phagocytic vacuoles surrounding an ingested bacterium in rabbit exudate heterophil leukocytes during phagocytosis. Using the differential centrifugation method, it is reported that neutrophilic granule fraction isolated from horse [7] and rabbit [8] blood leukocyte homogenate contained alkaline phosphatase.

As the ultrastructural localization of alkaline phosphatase in human neutrophil leukocytes has not yet been observed, the correlation between the enzyme and human neutrophil granules is quite unknown, although it is known that this enzyme appears in human neutrophil myelocytes and increases in number in mature neutrophils. Human neutrophil leukocytes have two types of granules [5]. The first (type A) (from 0.4–0.6 μm in length and 0.2 μm in width) are often elongated like rice grains and after staining with uranium acetate, are relatively clear. The second (type B) are smaller, 0.6–0.2 μm in diameter. After staining, they are very dense. Their population varies from one cell to another, but it seems that in man in the normal state there are about 70 of type A and 25 of the granules of type B.

In the present study it was found that the reaction positive granules were most likely to be specific granules. Furthermore, the localization of these granules appears to be somewhat different from acid phosphatase positive granules which are thought to belong to primary azurophil granules.

Acknowledgements. The author wishes gratefully to acknowledge the kind guidance and valuable suggestions of Prof. K. Ogaw and Dr H. Ma amara and to thank Prof. H. Fujii for his helpful advice in preparing the manuscript.

Summary

Ultrastructural cytochemical demonstration of alkaline phosphatase in human neutrophil leukocytes was carried out using Ma amara lead-citrate method. The reaction products were found to be clearly localized in some types of granules. The technical consideration and the localization of this enzyme were discussed.

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Author address Dr TOSHIYUKI NAKATSUI, Department of Internal Medicine, Research Institute for Nuclear Medicine and Biology Hiroshima University Kasumi-Cho, Hiroshima (Japan)

Acta haemat. 12: 281–293 (1960)

Institut für Balneologie und Klimaphysiologie (Direktor Prof. G. Göpfert) Medizinische Klinik (kom. Direktor Prof. J. Schirmeister) und Institut für medizinische Statistik und Dokumentation (Direktor Prof. E. Walter) der Universität, Freiburg/Br.

Über die Wirkung des Mittelgebirgsklimas und des Wetters auf das Blutbild[1]

H. Schulte-Wissermann, H. Hille, H. J. Jesdinsky,
H. Göpfert und K. G. v. Boroviczény

Es ist bekannt, dass bei einem Höhenaufenthalt oberhalb 2000 m der Erythrozyten- und Hämoglobingehalt des Blutes deutlich ansteigt [20, 27]. Über den Einfluss des Mittelgebirgsklimas auf das Blutbild findet man in der Literatur dagegen recht unterschiedliche Ansichten. Der Arbeitskreis um Verzár spricht dem Mittelgebirgsklima jegliche Wirkung auf das Blutbild ab [11, 27, 28]. Dies sei nach Ansicht jener Autoren auch nicht anders zu erwarten, da aufgrund der Barcroftschen O_2-Dissoziationskurven in diesen Höhen die Sauerstoffsättigung im Blut nur verhältnismässig wenig absinkt.

In neuerer Zeit wird jedoch von verschiedener Seite die von Miescher [21] schon vor 70 Jahren veröffentlichte Theorie wieder aufgegriffen, dass zwischen dem roten Blutbild und der Höhenexposition eine kontinuierlich progrediente Beziehung ohne irgendeinen markanten Schwellwert besteht. Diese Anschauung wird von Loewy [20] mit Nachdruck vertreten. Garthmann [13] wies nach, dass bei einem Aufenthalt in 1500 m die Erythropoese deutlich verstärkt wird und v. Muralt [22] beobachtete, dass bei einigen seiner gesunden Versuchspersonen ein Höhenwechsel von 500 m leichte, aber doch deutliche Blutbildveränderungen auslöste.

Die Blutbildveränderungen treffen jedoch nicht nur die roten Zellen, auch die Leukopoese wird durch eine Höhenexposition beein-

[1] Mit Unterstützung der Bundesversicherungsanstalt für Angestellte, der Landesversicherungsanstalt Baden und des Wirtschaftsministeriums Baden-Württemberg.

flusst BÜRKER *et al* [9] fanden bei ihren Untersuchungen auf der Schatzalp in 1850 m Höhe, dass die Gesamtzahl der Leukozyten etwas abnimmt KOLLER *et al* [18] teilten dagegen mit, dass in 3500 m Höhe die Leukozytenzahl ansteigt Das gleiche fand GÜNTHER [14] bei einem Aufenthalt in 1000 m KNOLL [17] untersuchte die Leukozytenwerte in der Höhe bei Arbeit Er konnte zeigen dass – im Gegensatz zum Verhalten bei Körperruhe – unter Arbeit die Zahl der neutrophilen Zellen wesentlich ansteigt, während die der Lymphozyten abfällt AMELUNG [1] *beobachtete bei Kuren in ca 400 m Höhe zwischen* der 2.–4 Woche eine Lymphozytose.

Auf Grund der verhältnismässig wenigen und sich zum Teil auch noch widersprechenden Literaturangaben schien es angebracht, sich erneut mit dem Problem der hämatologischen Wirkung des Mittelgebirgsklimas zu befassen. Bei der Durchführung derartiger Untersuchungen stösst man auf einige grundsätzliche Schwierigkeiten die vor allem dadurch ausgelöst werden dass jede Höhenexposition mit Veränderungen zahlreicher verschiedener Klima und Wetterfaktoren einhergeht

Material und Methodik

Um nach Möglichkeit vergleichbare und übersichtliche Bedingungen zu erhalten gingen wir bei unserem Experiment folgendermassen vor Während des Semesters wurden in Freiburg 30 gesunde Studenten genau voruntersucht und damit ein Kollektiv geschaffen, dessen biologische Daten bekannt waren. Aus diesem Kollektiv bildeten wir dann nach zufälliger Verteilung zwei Gruppen, von denen gleichzeitig die eine nach Bad Krozingen, 230 m ü N N die andere nach Hochenschwand, ca. 1000 m ü. N N. für 6 Wochen zu einem sanatoriumsartigen Aufenthalt geschickt wurden

In Bad Krozingen – nur 15 km von Freiburg entfernt herrscht praktisch das gleiche Klima wie in Freiburg. Die hier untergebrachte Gruppe war im wesentlichen als Kontrollgruppe anzusehen, denn die in Krozingen eventuell zu beobachtenden biologischen Veränderungen bei unseren Versuchspersonen können zur Folge des gegenüber Freiburg veränderten Milieus oder des Wettereinflusses der Grosswetterlage sein

Hochenschwand liegt nur 40 km ostwärts von Krozingen auf dem sanft nach Osten abfallenden Schwarzwaldplateau. Beide Orte werden gleichzeitig von Änderungen der Grosswetterlage getroffen. Auch hinsichtlich der Geopsyche [HELLPACH] weisen beide Orte gewisse Ähnlichkeit auf. Wir konnten also ziemlich sicher sein, dass eine Differenz in dem Befinden beider Gruppen als Folge des Höhenklimas gedeutet werden kann.

Die Versuche wurden von Mitte Februar bis Ende Mai 1968 durchgeführt. Zeitlich gliederte sich die Untersuchung in drei Phasen. Dem 6 Wochen dauernden Hauptversuch in den Kurorten ging eine dreiwöchige Voruntersuchung im Freiburger Institut voraus. Den Abschluss bildete eine sechswöchige Nachuntersuchung, bei der in der 1 und 2. sowie in der 5 und 6 Woche nach dem Ende des Hauptversuches gemessen wurde.

Der Gemeinde von Bad Krozingen und der Familie Porten in Hochenschwand gebührt unser Dank für die grosszügige Unterstützung des Untersuchungsprogrammes.

Am Ende des Versuches konnten die hämatologischen Daten von 27 Personen ausgewertet werden. Die Probanden waren während der Hauptversuchszeit gemeinsam in eigens hierfür bereitgestellten Häusern untergebracht. Die Verpflegung war an beiden Orten hinsichtlich Fett- und Eiweißverbrauch einheitlich, der Tagesablauf streng geregelt.

Alle Blutentnahmen erfolgten ausschließlich am Finger. Während der 3wöchigen Voruntersuchungen wurde einmal pro Woche morgens nüchtern, während der Hauptuntersuchung 3mal wöchentlich und in der Nachuntersuchungsperiode wiederum einmal wöchentlich Blut entnommen. Bei allen Untersuchungen wurden Doppelbestimmungen von zwei gesondert abgenommenen Blutproben vorgenommen. Während der gesamten Untersuchung nahmen bei den einzelnen Versuchspersonen jeweils die gleichen Hilfskräfte das Blut ab. Im einzelnen bestimmten wir folgende Werte

1. Die Gesamtzahl der Leukozyten und Erythrozyten wurde mit Hilfe eines elektronischen Zählautomaten (Coulter Counter D) nach der von Borowiczky, Gerike und Baumgartner beschriebenen Methode bestimmt.

2. Den absoluten Hämoglobingehalt ermittelten wir nach der internationalen Standardmethode (ICSH DIN 58931). Als Photometer diente ein Gerät der Fa. Eppendorf.

Die Bestimmungen von 1 und 2 führten wir zentral in Freiburg durch. Das Blut wurde für diesen Zweck unmittelbar nach der Entnahme mit einem PKW nach Freiburg transportiert.

3. Für die Hämatokritbestimmungen dienten hochtourige Zentrifugen (12000 U/min). Das Blut wurde in heparinisierten Glaskapillaren, deren exakte zylindrische Form vorher geprüft worden war, 10 min zentrifugiert.

4. Den mittleren Erythrozyten-Durchmesser ermittelten wir halometrisch nach der Methode von Borowiczky und Saffar in monochromatischem Licht von 550 nm am Blutausstrich.

5. Die Retikulozyten wurden nach der Methode von Heilmeyer gefärbt und von jedem Ausstrich 3000 Erythrozyten ausgezählt.

6. Für die Differentialblutbilder wurden frische Ausstriche gefärbt und von jedem Ausstrich 200 Zellen ausgezählt. Die statistische Auswertung der Meßergebnisse führte das Institut für medizinische Statistik und Dokumentation der Universität Freiburg durch. Dabei wurde für die varianzanalytische Behandlung der Kurvenläufe von der Methode der Orthogonalvergleiche Gebrauch gemacht, und zwar gewöhnlich von Orthogonalpolynomen sowie z.T. auch für Merkmale, von denen keine phasischen Schwingungen erwartet wurden, von Orthogonalsystemen zur Erfassung des Endes eines linearen Trends. Die Abhängigkeit einiger Merkmale des weißen Blutbildes von Wettereinflüssen wurde mittels der multiplen Regressionsrechnung untersucht.

Außer den Blutuntersuchungen wurden im gleichen Versuch noch Teste an Kreislauf, Atmung, Stoffwechsel, Wärmehaushalt und im ZNS durchgeführt. An beiden Versuchsorten registrierten wir fernerhin noch die üblichen meteorologischen Daten, wie Temperatur, Feuchte, Luftdruck, Strahlung, Wind, Niederschlag und Abkühlungsgröße.

Resultate

Rotes Blutbild. Vergleicht man die Zahl der Erythrozyten beider Kollektive (Abb. 1), dann fällt auf, daß beide Gruppen im zeitlichen Verlauf der Untersuchung teilweise gemeinsamen phasischen Schwankungen unterliegen. Die Zahl der roten Blutkörperchen weist drei Gipfel auf. Der erste Gipfel liegt zwischen der ersten und der zweiten Woche des Hauptversuches, der zweite in der vierten Woche und der

dritte in der sechsten Woche. Dieser Verlauf – mathematisch mit einem Polynom fünften Grades zu beschreiben – ist statistisch mit $p < 0{,}01$ gesichert. Diesem Trend folgen in der Höchenschwander Gruppe auch die Werte des Hämatokrites. Gemeinsam ist beiden Kollektiven auch eine deutliche Zunahme der Erythrozytenzahl im Verlauf der Hauptuntersuchung und der Abfall in der sich anschliessenden Nachuntersuchung.

Neben dem gemeinsamen Verlauf beobachteten wir jedoch auch Unterschiede zwischen beiden Gruppen. Es ist dies einmal ein gegenläufiges Verhalten zwischen der Tal- und der Höhengruppe in der ersten Untersuchungswoche, wo in der Höhe die Zahl der Erythrozyten stark ansteigt, während sie im Tal etwas abfällt. Zum anderen wächst die Zahl der roten Blutkörperchen im Gesamtverlauf der Hauptuntersuchungsperiode in Höchenschwand deutlich stärker an als in Krozingen. Der Unterschied ist mit $p < 0{,}001$ statistisch signifikant. In der Nachuntersuchung fällt die Zahl der Erythrozyten dann in beiden Kollektiven auf Werte ab, die unter dem Ausgangsniveau der Voruntersuchung liegen.

Recht eindrucksvoll ist das gegenläufige Verhalten der Retikulozyten während der Hauptuntersuchung (Abb. 1). Bei der Höhengruppe beobachtet man in der ersten Woche eine deutliche Retikulozytenausschwemmung. Mit 47200 Zellen/mm³ ist die Zahl der Retikulozyten am Ende der ersten Woche doppelt so hoch wie bei der Voruntersuchung. Die statistische Sicherung für den unterschiedlichen Verlauf an beiden Orten beträgt $p < 0{,}001$. Nach diesem primären Anstieg pendeln sich die Werte nach Art einer gedämpften Schwingung auf einen niedrigeren Pegel ein, der jedoch am Ende der Hauptuntersuchung noch immer über den Ausgangswerten liegt. Bei der Talgruppe beobachtet man demgegenüber, dass die Zahl der Retikulozyten nach einem primären Abfall ziemlich stetig bis zum Versuchsende ansteigt. Auch dieser Trend ist mit $p < 0{,}001$ signifikant.

Die mittleren Erythrozyten-Durchmesser verändern sich während der Hauptuntersuchung in beiden Orten annähernd gleichartig (Abb. 1). Sie weisen einen mit $p < 0{,}01$ gesicherten positiv quadrati-

Abb. 1. Mittelwertkurven der Erythrozyten, der Retikulozyten und der mittleren Erythrozytendurchmesser. I der Abszisse entspricht während der Vor- und Nachuntersuchung jeder Wert einem Wochenmittel, bei der Hauptuntersuchung dagegen jeweils einer Doppelbestimmung. Die Zahlen auf der Abszisse beziffern die Untersuchungswochen.

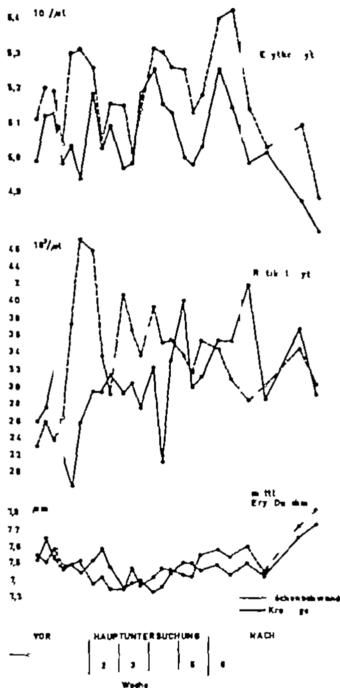


Abb. 1

schen Trend auf. Vier Wochen nach Ende des Hauptversuches werden die Erythrozyten beider Versuchsgruppen deutlich grösser. Ihr Durchmesser liegt bei der Krozinger Gruppe 0,16 µm, bei der Höchenschwander um 0,27 µm über den Werten der Voruntersuchung. Das entspricht einer Sicherung von $p < 0{,}001$.

Ebenso wie bei den roten Blutkörperchen ist auch bei den Hämoglobinwerten ein Höheneinfluss zu bemerken, obgleich die Befunde hier weniger eindrucksvoll sind. Nachdem in der Voruntersuchungsperiode bei beiden Gruppen praktisch gleich grosse Hämoglobinwerte gefunden wurden, liegen die Werte der Höhengruppe vom Ende der zweiten Hauptuntersuchungswoche – ausser bei einer Messung – immer über denen der Talgruppe. Der über dem gesamten Hauptversuch gemittelte Hämoglobingehalt liegt in Höchenschwand deshalb mit 15,8 g/100 ml um 0,19 g/100 ml über den Krozinger Werten. Dieser Unterschied ist aber statistisch nicht zu sichern.

Vom Hämatokrit wurde bereits erwähnt, dass er bei der Höchenschwander Gruppe den phasischen Veränderungen der Erythrozytenzahlen zeitlich folgt. Bei der Krozinger Gruppe ist diese Koppelung nur am Versuchsbeginn nachweisbar. Es ist darüber hinaus bemerkenswert, dass die Prozentzahlen in Höchenschwand während der Höhenexposition um 3,1% ansteigen (statistische Sicherung des unterschiedlichen Trendes $p < 0{,}01$), während die Werte der Krozinger Gruppe eher etwas absinken.

Weisses Blutbild. Bei der Gesamtleukozytenzahl ist das Absinken zu Beginn der Hauptuntersuchung und der anschliessende starke, ziemlich stetige Anstieg beider Gruppen auffallend (Abb. 2). Im Detail unterscheidet sich allerdings der Anstieg der Zellzahl in beiden Gruppen. Die Krozinger Gruppe zeigt eine fast gradlinige Tendenz, während die Höhengruppe in der 5. und 6. Woche auf einem Plateau verharrt. Die Unterschiede sind mit $p < 0{,}001$ statistisch gesichert. Bei dem Gang der Gesamtleukozytenzahl konnten wir den Einfluss des Wetters nachweisen. Das Auf und Ab der einzelnen Werte ist – vor allem bei der Höhengruppe – den Luftdruckschwankungen entgegengesetzt. Die negative Korrelation ist statistisch mit $p < 0{,}01$ gesichert. Der Abfall des Luftdruckes um 1 mm Hg löst im Durchschnitt einen Leukozytenanstieg um 75 Zellen/µl aus.

Im Differentialblutbild, das während der Voruntersuchung und am Ende der Nachuntersuchung recht einheitliche Werte aufweist, sieht man während des Hauptversuches bei verschiedenen Zellarten sowohl

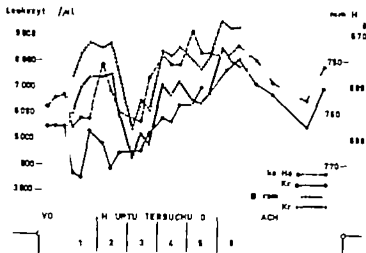


Abb 2. Mittelwertskurven der Leukozyten und der Tagesmittel des Luftdruckes. Abszissenteilung wie bei Abbildung 1 Da zwischen Leukozyten und Luftdruck eine umgekehrte Proportionalität besteht, ist die Ordinatenteilung für den Druck (rechts) von oben nach unten beschriftet. Hö. = Höchenschwander Gruppe, Kr = Krozinger Gruppe

recht deutliche Unterschiede zwischen der Höhen- und der Talgruppe als auch im zeitlichen Verlauf der Messgrössen während der Kur Sämtliche Werte für die segmentkernigen Granulozyten liegen in Höchenschwanden deutlich über den entsprechenden Zahlen im Tal (Abb 3) Die stabkernigen Granulozyten zeigen ein ähnliches Verhalten wie die bereits beschriebenen Retikulozyten Sie wurden in der Höhe zu Versuchsbeginn stärker ausgeschüttet als in Krozingen (Abb 4) Später im zweiten Drittel des Hauptversuches jedoch ändert sich die Situation da liegen die Werte der Talgruppe über denen von Höchenschwand Dieser unterschiedliche Trend zwischen der Höchenschwander und der Krozinger Gruppe ist mit $p < 0.01$ statistisch gesichert Im übrigen spiegelt sich auch bei den Stabkernigen die Abhängigkeit der Absolutwerte von den wetterbedingten Luftdruckschwankungen mit einer Signifikanz von $p < 0.01$ wider Des weiteren konnte eine direkte Korrelation zur Temperatur mit $p < 0.01$ gesichert werden. Hierbei ist allerdings zu bedenken dass die markantesten Luftdruckschwankungen in unserem Experiment unglücklicherweise mit den Zeitpunkten zusammenfielen an denen man nach Hildebrandt [15 16] mit phasenhaft ablaufenden regulativen Reak

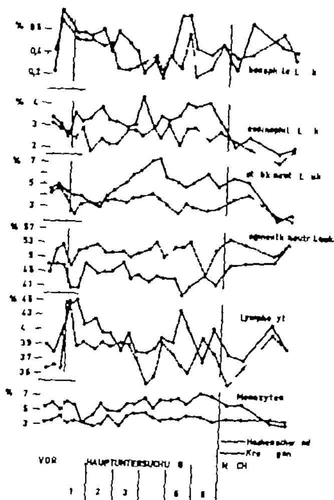


Abb. 3. Mittelwert des Differentialblutbildes in Prozenten. Abschnitteinteilung wie bei Abbildung 1

tionen als Antwort des Organismus auf den Klimareiz rechnen muss. Es wäre also auch möglich, dass die Zellzahländerungen auf die sogenannte Kurreaktion zurückzuführen sind.

Die Absolutwerte der Eosinophilen (Abb. 4) zeigen bei der Talgruppe im Gesamtverlauf des Hauptversuches eine ansteigende Tendenz, die man bei der Höchenschwander Gruppe nur in der ersten Hälfte des Hauptversuches beobachtet. Der Anstieg ist mit $p < 0{,}001$

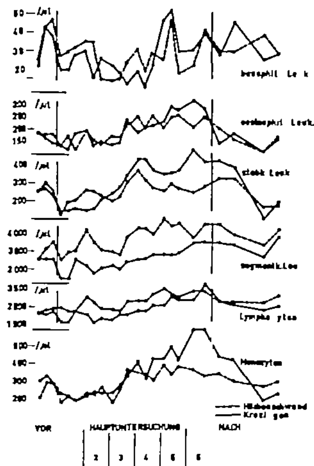


Abb. 4 Mittelwert des Differentialblutbildes in absoluten Werten pro µl. Abszisseneinteilung wie bei Abbildung 1.

gesichert. Über diesen Trend hinaus spiegelt sich auch bei den Eosinophilenzahlen der Einfluss des Wetters wider. Hier korrelieren auch die Werte der Krozinger Gruppe negativ mit dem Luftdruck. Entsprechend der niedrigeren Gesamtgranulozytenzahl in Krozingen ist der prozentuale Eosinophilengehalt des Blutes im Tal während des Hauptversuches grösser als in der Höhe. Die statistische Sicherung dieses Unterschiedes beträgt $p < 0{,}05$

Die Lymphozyten verhalten sich während des Hauptversuches ähnlich wie die Segmentkernigen. Bei beiden Gruppen steigt die Zellzahl im Verlauf der Hauptuntersuchung etwas an, wobei die Höhengruppe der Talgruppe wieder etwas vorauseilt. In der Nachuntersuchungsperiode sinken die Werte wieder auf das Ausgangsniveau ab. Bei der Höchenschwander Gruppe kann man darüber hinaus eine Wetterabhängigkeit wie bei den Segmentkernigen erkennen, deren Sicherung $p < 0{,}01$ ist.

Diskussion

Die Ergebnisse unserer Verlaufsuntersuchung zeigen, dass sowohl das Höhenklima als auch die akute Wettersituation einen Einfluss auf das Blut ausübten. Darüber hinaus löste aber bei unseren Studenten auch der *Milieuwechsel* vom angespannten Semesterbetrieb in die Geborgenheit eines Sanatoriumsaufenthaltes Veränderungen am Blutbild aus. Wie anders sollte man sich sonst die systematische Befundsänderung bei der Krozinger Gruppe während des Hauptversuches erklären, wo sich die Messwerte gegenüber der Vor- und Nachuntersuchung deutlich abheben.

Versuchen wir nun die Effekte dieser drei Wirkungskomponenten voneinander zu trennen. Der Anstieg der Erythrozyten zu Beginn des Höhenaufenthaltes im Mittelgebirge ist gesichert, wie dies vom Hochgebirge bereits bekannt ist. Die gleichzeitig einsetzende Retikulozytose beweist, *dass es sich hierbei um einen echten hämatopoietischen Reiz* handelt und nicht nur um die Ausschüttung von Erythrozyten aus Blutspeichern, wie dies von Schönholzer und Lüthi [24] angenommen wird.

Die Ähnlichkeit der Reaktion mit den vom Hochgebirge her bekannten Vorgängen legt es nahe, in beiden Fällen als Ursache die Verminderung des Sauerstoffpartialdruckes anzunehmen. Dabei kann vorerst nicht entschieden werden, ob die Druckminderung direkt als auslösendes Agens fungiert oder ob sekundär der durch jene ausgelöste, geringfügige Abfall der Sauerstoffsättigung des Blutes verantwortlich gemacht werden muss. Beide Wege scheinen uns möglich. Dagegen halten wir es nicht für angezeigt, bei der Stimulation der Erythropoese im Mittelgebirge nach einem anderen auslösenden Agens als im Hochgebirge zu suchen.

Neben diesen Reizen auf die Erythropoese laufen bei einem Höhenaufenthalt in den ersten Tagen, wie BECK *et al.* [4] nachweisen konnten, destruktive Prozesse unbekannter Genese am Erythrozyten ab, die sich in einer Realstenzminderung der roten Blutkörperchen in einer Bilirubinämie [12] und Urobilinogenurie [27] äussern. Es ist möglich, dass die etwas niederen Erythrozytenwerte bei der ersten Blutentnahme unserer Höhengruppe auf derartige Prozesse zurückgeführt werden können. Ähnliche Befunde teilten DIRNAGEL *et al.* [10] von einer Untersuchung in Oberstdorf (850 m ü. N. N.) mit.

Interessant ist der Verlauf der Retikulozytenzahl in Höchenschwand während des Hauptversuches, denn hier sieht man, wie sich ein aus dem steady state gebrachtes Regelsystem in Form einer gedämpften Schwingung auf ein neues Niveau einstellt. Die Schwingungsdauer beträgt hierbei von Maximum zu Maximum etwa 10–12 Tage. Auf derartige Einschwingungsvorgänge, die man lange Zeit übersehen hat, machte vor allem HILDEBRANDT [16] aufmerksam.

Wesentlich schwieriger sind die phasischen Verläufe der Erythrozytenzahlen zu deuten, die im Prinzip in beiden Gruppen gleichartig ablaufen, bei denen jedoch – wenn man von der ersten Woche absieht – ganz deutlich eine Phasenverschiebung zwischen Höchenschwand und Krozingen festzustellen ist, dergestalt, dass die Werte in Krozingen immer etwas vor denen von Höchenschwand abfallen. Die synchron mit den Erythrozytenzahlen veränderten Hämatokritwerte von Höchenschwand lassen an Hydratationsvorgänge als auslösende Ursache für diese Phasen denken. Dem stehen jedoch die Befunde von Krozingen entgegen, wo kein Zusammenhang zwischen diesen beiden Messgrössen besteht. Es liess sich auch keine Korrelation dieser Phasen mit irgendeinem meteorologischen Befund nachweisen. Dagegen fiel auf, dass die Ausscheidung der 17-Hydroxy-Verbindungen im Harn bei unseren Probanden in Höchenschwand und Krozingen ähnlich synchronen Schwankungen unterworfen war [23]. Wir müssen diese phasischen Gänge der Erythrozytenwerte in jene eigenartigen Schwingungsvorgänge einreihen, die in der physikalischen Therapie bei den verschiedensten Kuren bereits von zahlreichen anderen biologischen Funktionen her bekannt sind [16]. Eine stichhaltige Erklärung für solche Phänomene konnte hierfür bisher noch nicht gegeben werden.

Einer kurzen Erwähnung bedarf noch die in der zweiten Hälfte des Hauptversuches verbesserte Erythropoese der Talgruppe. Hier kommen wohl vor allem zwei Komponenten zum Zug. Einmal wirkt – das

konnte durch gleichzeitig mit den hämatologischen Studien durchgeführte Untersuchungen am Kreislauf, Stoffwechsel und ZNS nachgewiesen werden – der einfache Milieuwechsel auch im gleichen Klima wie ein Streß. Diesem Streß schloß sich dann eine Phase echter Erholung im Sanatorium an. Schließlich war die Ernährung unserer *Probanden während des Hauptversuches hochwertiger als während* der Semesterzeit, so daß auch über den Umweg eines verbesserten Eisen- und Eiweißangebotes ein günstiger Effekt auf die Blutbildung ausgeübt werden konnte.

Im roten Blutbild konnten wir nur Veränderungen beobachten, die durch den Einfluß des Klimas und des Milieus hervorgerufen wurden. Im weißen Blutbild dagegen zeigte es sich, daß auch akute Wetterveränderungen das morphologische Substrat beeinflussen können. Die bisher in der Literatur beschriebenen Befunde hinsichtlich des Verhaltens der Gesamtleukozytenzahl bei Klimakuren widersprechen sich teilweise. *So fand* GÜNTHER *[14] bei Untersuchungen an Arthritikern* in Bad Gastein (1000 m) einen signifikanten Anstieg der Zellzahlen während eines vierwöchentlichen Aufenthaltes. Ähnliches teilen KOLLER *et al.* [18] aus 1600 m Höhe mit. Diese Autoren fanden, daß mit der Leukozytose eine vermehrte Ketosteroid- und Corticoidausschüttung einhergeht. BÖRKER *et al* [9] teilen dagegen von einer vierzehntägigen Beobachtung in 1850 m das Gegenteil mit. Wir können uns mit unseren Befunden hinsichtlich der Gesamtleukozytenzahl grundsätzlich den Beobachtungen von GÜNTHER und KOLLER *et al.* anschliessen, denn wir fanden daß nach einem primären Abfall der *Leukozytenzahl beide Kollektive einen signifikanten* Anstieg der Gesamtleukozytenzahl aufwiesen. In der Art, wie dieser Anstieg verläuft, unterscheiden sich jedoch die Gruppen voneinander Während bei der Talgruppe die Zellzahl von der zweiten Woche an stetig und kontinuierlich zunimmt, sind diesem – auch bei der Höhengruppe vorhandenen Anstieg – bei den Höchenschwander Probanden deutlich wetterabhängige Schwankungen überlagert.

Dieser Einfluss des Wetters wird vor allem bei einer eingehenden Analyse des Differentialblutbildes deutlich. Zur Analyse dieses Einflusses wurden die Merkmale des weissen Blutbildes beider Orte mit der *multiplen Regressionsanalyse* getrennt untersucht. Ausser den meteorologischen Daten von Temperatur Luftdruck, Niederschlag und Strahlung berücksichtigten wir noch den Zeitfaktor durch Hinzunahme der Nummern des Messtages und deren Quadraten, um so

einen allgemeinen Trend während der Hauptversuchszeit von wetterbedingten Effekten abtrennen zu können. Die negative Korrelation zwischen dem Luftdruck und den Gesamtleukozyten wurde bereits erwähnt. Darüber hinaus zeigte sich aber in Höchenschwand bei den stabkernigen Granulozyten ein mit $p < 0.05$ signifikant senkender Einfluss der Temperatur auf die Zellzahl. In Krozingen hinwieder sank die Zahl der Monozyten mit steigender Temperatur ab (Signifikanz $p < 0.05$). Die eosinophilen Granulozyten zeigten eine positive Partialkorrelation ($p < 0.05$) zur Strahlungsintensität, wenn man die gemittelten Partialkorrelationen für beide Orte zusammenfassend betrachtet.

Die aufgezählten Daten zeigen, dass das Wetter einen Einfluss auf die Zusammensetzung des weissen Blutbildes ausüben kann, der in der Höhe stärker ist als im Tal. Das mag daran liegen, dass die Höhengruppe während der Hauptversuchszeit einem höheren Reizpegel ausgesetzt war und dadurch eine Sensibilisierung erfolgte, auf meteorologische Reize stärker zu reagieren.

Bei den Lymphozyten konnte keine Beeinflussung durch das Wetter festgestellt werden. Hier steigt die Zellzahl im Verlauf der Hauptuntersuchung bei beiden Gruppen an. Wir sehen hierin eine Bestätigung der von AMELUNG [1] beschriebenen Akklimatisationslymphozytose.

Schliesslich scheint es uns noch erwähnenswert, dass der Verlauf der Retikulozyten und der Stabkernigenzahlen der Höhengruppe im Hauptversuch gewisse Ähnlichkeiten zeigt.

Man muss sich vor einer zu speziellen Interpretation unserer Ergebnisse hinsichtlich des Wettereinflusses auf das Blutbild hüten. Wenn gesagt wurde, dass einem bestimmten Luftdruckanstieg eine definierte Zellzahlveränderung entspricht, dann soll dies nicht heissen, dass mit der Drucksenkung allein diese Veränderung reproduzierbar sein müsste. Derartige Kausalzusammenhänge mit einzelnen Wetterfaktoren sind bis jetzt experimentell nur selten nachgewiesen worden. Die Biometeorologie konnte nur in Ausnahmefällen zeigen, welche meteorologischen Einzelfaktoren einen spezifischen Einfluss auf biologische Funktionen ausüben. Es kommt im Wettergeschehen nie vor, dass sich nur eine meteorologische Komponente allein verändert. Es sind immer sehr komplexe Vorgänge, bei denen zahlreiche Einzelfaktoren in systematischer Weise miteinander verknüpft sind. Wir möchten deshalb unsere Befunde dahingehend deuten, dass die signifikanten Be-

ziehungen zwischen den Blutwerten und den einzelnen Wetterfaktoren als Zeichen dafür anzusehen sind, dass ein meteorologischer Komplex – sei es Schönwetter, Regen oder Frontendurchgang – eine Rückwirkung auf das biologische Geschehen ausübt, ohne dass wir im einzelnen wissen, welche Detailfaktoren hierbei die entscheidende Rolle spielen.

Zusammenfassung

An 2 Kollektiven mit 13 bzw. 14 Versuchspersonen, wurde der Einfluss des Klimas auf das Blutbild untersucht. Schon in 1000 m Höhe nach einem Höhenanstieg von ca. 750 m erfolgte eine deutliche Stimulierung der Erythropoiese. Als auslösende Ursache wird der Einfluss des verminderten O_2-Partialdruckes in der Höhe angenommen. Die Erythrocytenwerte wiesen darüber hinaus im Verlauf der Kur phasische Schwankungen in einem Rhythmus von 9–10 Tagen auf, während die Zahl der Retikulocyten in der Höhe nach überschiessender Reaktion auf einen höheren Wert eingestellt wurde. Sowohl im Tal als auch in der Höhe konnte eine verstärkte Leukopoiese nachgewiesen werden. Vor allem wurde der Einfluss des Wetters deutlich. Die Gesamtzahl der Leukocyten sank bei steigendem Luftdruck. Noch deutlicher reagierten die stabkernigen Granulocyten und die Monocyten auf wetterbedingte Temperaturänderungen. Ausserdem bestand eine positive Korrelation zwischen der Zahl der eosinophilen Granulocyten und der Strahlungsintensität.

Summary

The influence of climate on the blood count was studied in two groups of 13 and 14 subjects respectively. Erythropoiesis is strongly stimulated at a height of 1,000 m after an ascent about 750 m. The causative mechanism is considered to be the reduced partial pressure of oxygen in the atmosphere at this altitude. Furthermore, the erythrocyte values fluctuate in cycles of nine to ten days during the course of the treatment, while the number of reticulocytes, after an initial exaggerated response to the high altitude, settled down at a raised level. Increased leukopoiesis was found in both the mountain and the valley group. In particular, the influence of the weather was obvious. As the pressure rose, the total number of leukocytes fell. Staff cells and monocytes were even more sensitive to temperature changes resulting from the weather. The number of eosinophilic granulocytes was also correlated positively with the intensity of radiation.

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Adresse der Autoren: Dr. H. Schulte Wissermann, Doz. Dr. H. Hille, Prof. Dr. H. Göpfert, Institut f. Klimaphysiol. d. Univ., H. Herder-Strasse 7, *D-78 Freiburg*; Dr. H. J. Jesdinsky, Institut f. med. Statistik d. Univ., St. Meier-Strasse 26, *D-78 Freiburg*; Dr. K. G. Boroviczény, Med. Univ.-Klinik, Hugstetter Strasse 55, *D-78 Freiburg* (Deutschland)

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I Medizinische Klinik und Poliklinik der Johannes-Gutenberg-Universität
(Direktor: Prof. H. P Wolff) Mainz

Der Einfluß dysproteinämischer Seren auf die Resistenz normaler Erythrozyten

F Gramlich und F Kirchhoff

Die Ursachen extrakorpuskulär bedingter hämolytischer Anämien sind vielfältig Während hämolytische Syndrome relativ häufig beobachtet werden, etwa in Form der sogenannten symptomatischen hämolytischen Anämien bei Infektionskrankheiten malignen Tumoren, bei Leberzirrhosen und rheumatischen Erkrankungen, gelingt der Nachweis immunogener Autohämaggresine nur *relativ selten* [5] Als weiterer extrakorpuskulär wirksamer Hämolysefaktor konnte zwar die erythroklastische Sequestration des retikuloendothelialen Systems *in Milz* und *Leber* gesichert werden, doch wurde gleichzeitig als allgemein gültiges pathogenetisches Prinzip hämolytischer Erkrankungen erkannt, dass eine Primärschädigung des Erythrozyten der Blutmauserung durch das retikuloendotheliale System vorausgehen muss [1 2] Als schädigende Agentien, die über das Blutplasma an die roten Blutzellen herantreten fanden sich neben den Autohämaggresinen und einigen anderen Immunkörpern bisher lediglich Stoffe mikrobieller Herkunft [6] Einen weiteren Hinweis auf die Pathogenese extrakorpuskulärer hämolytischer Anämien lieferten die Mitteilungen von Furchgott und Ponder [3 4] über die Existenz eines albuminähnlichen «antispheringous factor» der die Oberfläche der roten Blutkörperchen filmartig überziehen und die Membraneigenschaften bzw die Stabilität der typischen Erythrozytenform mitbestimmen soll. Tatsächlich konnte durch wiederholte Erythrozytenwaschungen mit isotoner Glukoselösung gezeigt werden, dass vom physiologischen Plasmaeiweissmilieu formerhaltende Wirkungen auf

die roten Blutkörperchen ausgehen [15]. Dass eine Dysproteinämie die Membraneigenschaften der roten Blutkörperchen im Sinne einer Forminstabilität und Oberflächenschädigung mit konsekutiver Sequestration zu beeinflussen vermag, konnte bisher allerdings nicht bewiesen werden [7].

Auf Grund eigener Untersuchungen über den Plasmaproteinfilm der Erythrozyten [11] haben wir den Einfluss dysproteinämischer Seren auf die Resistenz roter Blutkörperchen erneut geprüft.

Methode

Erythrozyten. Aus Blut gesunder Spender der Blutgruppe O Rh+ wurden unter Blutbankbedingungen Blutkonserven hergestellt. Die Verwendung normaler Spendererythrozyten sollte Einflüsse korpuskulärer Minderwertigkeiten, die Wahl der Blutgruppe Isoagglutinations-Reaktionen ausschliessen. Die Weiterverarbeitung erfolgte am Entnahmetag durch 3maliges Waschen in der Zentrifuge mit dem 6fachen Volumen kalter physiologischer Kochsalzlösung. Die Leukozytenschicht wurde abgesaugt. Für die Inkubationsansätze wurde das Erythrozytensediment nach der letzten Waschung (MRZ = 1250 × g, 5 min) unverzüglich benutzt.

Seren. Zur Untersuchung gelangten Nüchternseren von 35 jugendlichen Personen (Kontrollgruppe) und Proben von 67 Patienten bestimmter Krankheitsgruppen (Tab. I). Die Seren wurden durch Zentrifugation vom Blutkuchen getrennt, vor der Inkubation mit den Testerythrozyten 30 min bei 56°C im Wasserbad inkubiert, um hämolysierende Komplementwirkungen auszuschalten, und elektrophoretisch analysiert. Die Pherogrammwerte der Kontrollgruppen lagen im Normbereich. 41 Proben der pathologischen Seren wiesen über 20 rel% α_1- und α_2-Globuline auf, und 26 pathologische Seren enthielten über 30 rel% γ-Globuline (Tab. I).

Versuchsansatz. In Wassermann-Röhrchen wurden Erythrozyten und Seren unter sterilen Bedingungen im Volumverhältnis 2:1 vermischt und verschlossen 20 h lang im Wasserbad bei 37°C inkubiert. Damit wurde beabsichtigt, den sogenannten Endoparaseeffekt, unter dem die Blutmauserung in der roten Pulpa der Milz stattfindet, auf den in-vitro-Ansatz zu übertragen. Blutproben für die Resistenzprüfung wurden nach 1 und 20 h entnommen.

Resistenzprüfungen. a) Mechanische Resistenz nach SCHUBOTHE [14]; b) osmotische Resistenz gegen 0,10–0,85 g% Kochsalzlösungen, gemessen 30 min nach Inkubation bei Zimmertemperatur; c) Spontanhämolyse im Serum der Proben (Extinktionsmessung bei λ = 546 nm, D = 10 mm, gegen uninkubiertes Leerserum).

Auswertung. Die mechanische Hämolyse wurde in % der Totalhämolyse errechnet. Die osmotische Hämolyse wurde für die einzelnen Kochsalz-Konzentrationen in % der Totalhämolyse bei 0,1% Kochsalz ermittelt. Für die Auswertung wurden der Hämolysegrad bei 0,5% Kochsalz und die Kochsalzkonzentration der 50% Hämolyse benutzt. Bei der Spontanhämolyse wurden die Logarithmen der Extinktionswerte miteinander verglichen.

Die statistische Bewertung der Ergebnisse erfolgte zunächst mittels BARLETT-Test [12] und der einfachen Varianzanalyse [13], wobei 3 Gruppen (Normalseren, Seren mit α- und Seren mit γ-Globulinvermehrung) miteinander verglichen wurden. Da die Varianzanalyse nur Gruppenunterschiede feststellt, wurde zur Klärung der Frage, welche Gruppen sich voneinander abheben, der DUNCAN-Test [16] für Stichproben herangezogen.

Tabelle I Relative Prozentwerte der α- und γ-Globuline bei den 6 untersuchten Krankheitsgruppen

Patienten		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Metastasierende Karzinome 14	α	28	–	26	–	24	23	23	31	24	21	29	21	22	
	γ	–	31	–	36	–	32	–	30	–	–	30	–	–	31
2. Hämoblastosen (Lymphogranulomatose, Leukosen, Myelome, Retikulosen) 16	α	27	31	28	–	24	23	–	32	23	21	–	27		
	γ	–	–	–	32	–	–	36	–	–	–	31	–	33	31
3. Akute Infekte (Pneumonie, Pleuritis, Cholecystitis) 9	α	30	28	36	–	28	28	23	23	24					
	γ	–	–	40	30	–	–	–	–	–					
4. Leberzirrhosen und chronische Hepatitiden 9	α	–		–	–	–	–	–	–	–					
	γ	45	31	38	40	31	32	35	32	32					
5. Akute rheumatische Erkrankungen (akute Polyarthritis, PCP) 7	α	23	22	22	23	23	21	24							
	γ	–	–	–	–	–	–	–							
6. Kompensierte Nierenerkrankungen (chronische Glomerulo- und Pyelonephritis, Nephrosen, Zystennieren) 12	α	25	22	23	26	27	22	30	30		27	–			
	γ	–	–	–	–	–	–	–	–	33	–	42	31		

α-Globuline < 18, γ-Globuline < 24 rel%

Ergebnisse

1. Erythrozytenresistenz und Dysproteinämie. In Tabelle II sind die statistisch ausgewerteten Ergebnisse der mechanischen und osmotischen Hämolyseprüfung sowie der Spontanhämolyse der Testerythrozyten unter dem Einfluss von Normalseren und Dysproteinämien wiedergegeben. Die *mechanische Resistenz* wird durch dysproteinämische Seren mit α- oder γ-Globulinvermehrungen nicht signifikant verändert ($p > 0.05$). Dagegen nimmt die *osmotische Resistenz* unter dem Einfluss dysproteinämischer Seren ab. Die Steigerung der osmotischen Hämolyse bei einer Kochsalz-Konzentration von 0,5% wird gleichermassen von den α- und γ-Globulin-reichen Seren bereits nach 1stündiger, deutlicher aber nach 20stündiger Inkubation hervorgerufen. Die

Tabelle II Varianzanalyse. Irrtumswahrscheinlichkeit (p), mit der sich die hämolysierende Potenz dysproteinämischer Seren von Normalseren unterscheidet

		1 h	20 h
Mechanische Resistenz	α-Glob. > 20 rel%	> 0,05	> 0,05
	γ-Glob. > 30 rel%	> 0,05	> 0,05
0,5% NaCl	α-Glob. > 20 rel%	< 0,01	0,001
Osmotische Resistenz	γ-Glob. > 30 rel%	< 0,01	0,001
50% Hämolyse	α-Glob. > 20 rel%	> 0,05	0,001
	γ-Glob. > 30 rel%	> 0,05	0,001
Spontanhämolyse	α-Glob. > 20 rel%	< 0,01	> 0,05
	γ-Glob. > 30 rel%	< 0,01	> 0,05

50%ige Hämolyse wird nach 20stündiger Inkubation mit den dysproteinämischen Seren schon bei signifikant höheren Kochsalzkonzentrationen erreicht als bei Normalseren.

Während bei der 50% Hämolyse nur der 20-Stunden-Wert einen signifikanten Unterschied zwischen dysproteinämischen und normalen Seren erkennen lässt, war bei der *Spontanhämolyse* lediglich nach 1stündiger, nicht aber nach 20stündiger Inkubation ein vermehrter lytischer Einfluss der dysproteinämischen Seren nachweisbar.

Besonders bemerkenswert ist, dass die Steigerung der Hämolyse durch Seren mit α-Globulinvermehrung als auch durch Seren mit γ-Globulinvermehrung in gleichem Masse erzielt wurde.

2. *Auswertung nach Krankheitsgruppen.* Für die Untersuchungen wurden Krankheitsgruppen (Tab. I) ausgewählt, bei denen einerseits extrakorpuskuläre Hämolyseeinflüsse häufig beobachtet werden und die andererseits mit stärkergradigen Dysproteinämien einhergehen. Es konnte varianzanalytisch festgestellt werden, dass zwischen den Hämolysegraden der untersuchten Krankheitsgruppen keine Unterschiede bestehen.

Diskussion

Der Versuchsansatz mit hoher Erythrozytenkonzentration und Inkubation bei 37°C bis zu 20 h beabsichtigte, die physiologischen Verhältnisse der Blutmauserung in der Milz im Experiment nachzuahmen. Die Erythrozyten waren während der *in vitro*-Inkubation auch ähnlichen Stoffwechselbelastungen ausgesetzt wie in der Milz

[17] Die Überprüfung der Wasserstoffionen Bikarbonat und Glukosekonzentration sowie der Viskosität (Ostwaldt Kapillarviskosimeter $\varnothing = 0.05$ cm) und der Filtrierbarkeit (Sartorius-Membranfilter Göttingen Porendurchmesser 5 μm) des Versuchsansatzes während der 20stündigen Inkubation ergab charakteristische Veränderungen, die unterschiedslos in den Kontroll und dysproteinämischen Proben angetroffen wurden. Mit Utilisation der Glukose durch die erythrozytäre Glykolyse wurde zunehmend Laktat gebildet, wodurch Bikarbonat verbraucht und das pH im Ansatz absank. Die Säuerung des Blutes bewirkte ihrerseits einen sphärischen Gestaltwandel der Erythrozyten, erkenntlich am Ansteigen des Hämatokrits. Gleichzeitig nahm die relative Viskosität des Blutes zu und die starren Sphärozyten passierten die Poren des Filters nicht mehr.

Wie die Versuchsergebnisse in Tabelle I zeigen, wirken sich die Stoffwechselbelastungen auf Erythrozyten im dysproteinämischen Milieu, gemessen an der osmotischen Belastbarkeit und der Spontanhämolyseneigung signifikant stärker aus als in den euproteinämischen Kontrollansätzen. Bemerkenswert ist dabei, dass die vermehrte Hämolyse von Seren mit α und γ-Globulinvermehrung ohne Unterschied herbeigeführt wird. Die mechanische Resistenz der Erythrozyten dagegen wies bei grosser Streubreite der Messwerte in allen Gruppen keine Unterschiede auf. Auch von Hämolyseuntersuchungen bei immunhämolytischen und korpuskulär bedingten, hämolytischen Anämien ist bekannt, dass die Empfindlichkeit geschädigter Erythrozyten gegen mechanische Einflüsse weniger gut darstellbar ist als gegen osmotische Einwirkungen [1].

Im Hinblick auf die Hämolyseursache bleibt nach Ausschluss korpuskulär oder durch «Komplement» bedingter Hämolyseeinflüsse durch den Versuchsansatz die Frage, ob das dysproteinämische Serummilieu die Membranstabilität der Erythrozyten beeinflussen kann. Da die hämolytische Wirksamkeit von Gewebshämolysinen oder mikromolekularen Serumbestandteilen wenig wahrscheinlich ist [1], ist zu diskutieren, ob bei Dysproteinämien Normabweichungen in der Zusammensetzung des Plasmaproteinfilms an der Oberfläche der Erythrozyten deren Membraneigenschaften verändern.

Durch Anwendung radioaktiv markierter reiner Plasmaproteine war es uns [6] und anderen Autoren [8, 9] möglich, die Existenz eines Plasmaproteinfilmes an der Erythrozytenoberfläche zu beweisen und darüberhinaus auch die Zusammensetzung des Erythrozytenfilmes abzuschätzen sowie die Bedingungen zu studieren, mit denen sich die Serumproteine an die Erythrozytenmembran binden. Auf Grund unterschiedlich hoher Bindungsneigung

der einzelnen Plasmaproteine zur Erythrozytenoberfläche unterscheidet sich die prozentuale Zusammensetzung der Proteine im Serum grundsätzlich von der Zusammensetzung am Erythrozyten. So sind beispielsweise γG-Globulin, Fibrinogen, α-Lipoprotein, α_2-Makroglobulin und Präalbumin auf Kosten insbesondere des Albumins und des Transferrins auf der Erythrozytenoberfläche um das 1,2 3,8fache angereichert [10]. Die Zusammensetzung des Plasmaproteinfilmes ist unter dysproteinämischen Bedingungen gegenüber der Norm verändert [11], wobei Auswirkungen auf die Membraneigenschaften der Erythrozyten postuliert werden können [6].

Die Veränderungen der Serumeiweisszusammensetzung betraf in den vorgelegten Versuchen die α- oder die γ-Globuline. Unter ihnen ist eine besonders hohe Erythrozytenbindungsneigung der Lipoproteine und Makroglobuline des α-Bereiches bzw. der γG-Globuline nachgewiesen worden [6]. Dass der Plasmaproteinfilm die Membraneigenschaften der Erythrozyten mitbestimmt, lässt sich aus experimentellen Befunden ableiten, wonach die Plasmaproteine mit prinzipiell gleichen, wenn auch geringeren physikalischen Kräften als immunogene Hämaggresine mit der Erythrozytenoberfläche in Kontakt treten [6]. Im Gegensatz zu der Membranschädigung der spezifischen Hämaggressine [1] wirkt sich die Bindung der Plasmaproteine an der Erythrozytenoberfläche unter normalen Bedingungen stabilisierend auf die roten Blutkörperchen aus [7]. Unter dysproteinämischen Bedingungen kann vermutlich durch Änderung der normalen Zusammensetzung des Plasmaproteinfilmes an der Erythrozytenoberfläche eine Störung der Membranstabilität eintreten, die die Lebenszeit der Erythrozyten verkürzt. Gerade der zunächst überraschende Befund, dass die Hämolyseneigung der Erythrozyten unabhängig von den Krankheitsgruppen sowohl durch Vermehrung der α- als auch der γ-Globuline gleichgerichtet zunimmt, stützt die Hypothese, dass die Membranschädigung der Erythrozyten unspezifisch erfolgt und über die Änderung der Zusammensetzung des regulären Plasmaproteinfilmes bei stärkergradigen Dysproteinämien und bei Paraproteinämien zustandekommt.

Zusammenfassung

Die Inkubation normaler Erythrozyten mit dysproteinämischen Seren führt zu einer Steigerung der Spontanhämolyse und zu einer Herabsetzung der osmotischen Resistenz. Die mechanische Resistenz wird dabei nicht verändert. Der Einfluss dysproteinämischer Seren wird auf eine Störung der normalen Zusammensetzung des Plasmaproteinfilmes an der Oberfläche der roten Blutkörperchen und damit auf eine Beeinflussung der Membraneigenschaften zurückgeführt.

QUICK [37], partielle Thromboplastinzeit nach LANGDELL [23] mit Kaolinzusatz (Reagens der Behringwerke) Heparintoleranztest nach MARBET und WINTERSTEIN [29] mit 0,5 und 0,2 E Heparin pro ml Ansatz, Prothrombinverbrauchstest nach SOULIER [40], Thrombinzeit (mit 5 E Thrombin/ml) Thrombelastogramm nach HARTERT [16] Fibrinogen nach CLAUSS [10], Faktor VIII nach BARROW *et al.* [1].

2. *Testung der Thrombozytenfunktion.* Thrombozytenfaktor 3 nach DEUTSCH, Thrombozytenfaktor 4 nach DEUTSCH [12] Retraktion nach BAUDOUX [8] und BENTHAUS [3], Thrombozytenausbreitung nach BAUDOUX und BÜRGI [6], Thrombozytenadhäsivität nach BAUDOUX [7]. Die Thrombozytenaggregation wurde photometrisch nach BORN [5] bestimmt. 1,0 ml plättchenreiches Plasma, das durch Zugabe von plättchenarmem Plasma auf eine konstante Thrombozytenzahl von 80000 eingestellt wurde, wurde zunächst 2 min auf 37° C vorgewärmt und dann in eine thermostatierte Küvette eines *EEL-Photometers (Evans-Electroselenium-Ltd., Halstead, Essex)* gegeben. Die Lichtdurchlässigkeit wurde mittels eines Beckmannschreibers kontinuierlich registriert. Die aggregierenden Substanzen (ADP Adrenalin und Kollagen) wurden mittels Mikropipetten zugegeben (Volumen 10–100 µl) Als Maß der Aggregation wurde die Initialgeschwindigkeit (V) der Extinktionsänderung angegeben [2], wobei V = mm/mm.

Die Aufnahme von markiertem Serotonin wurde in folgender Weise gemessen 9,0 ml plättchenreiches Plasma wurden mit 1,0 ml ^{14}C markiertem Serotonin (Amersham, 5 µg/ml) in silikonierten Glasröhrchen unter Schütteln bei 37° C inkubiert. In Abständen von 15 min wurden 1,5 ml Inkubationsmischung entnommen, 5 min bei 2500 rpm zentrifugiert, das Plättchensediment in 1,0 ml EDTA-NaCl resuspendiert und neuerlich bei 2500 rpm 5 min zentrifugiert. Das Sediment wurde mit 0,8 ml NCS versetzt und als Szintillationsflüssigkeit Toluol zugegeben. Die Bestimmung der Radioaktivität erfolgte mit einem Liquidszintillationszähler (Packard)

3. *Die Bestimmung der Enzymaktivitäten und der Substratkonzentrationen* in den Thrombozyten erfolgte mit den früher beschriebenen Methoden [32].

4. *Ultrastrukturelle Untersuchungen der Thrombozyten.* Zur elektronenmikroskopischen Darstellung der Thrombozyten wurden zwei Methoden angewandt: Bei Methode A wurden 8 ml Venenblut des Patienten mit 1 ml EDTA, 0,5 ml Glutaraldehydkakodylat (5% Glutaraldehyd in 0,1 mol. Kakodylatpuffer pH 7,3) und 0,5 ml 5%iger Dextrose in einem silikonierten Zentrifugenröhrchen gemischt und bei 3000 Umdrehungen 10 min bei Zimmertemperatur zentrifugiert. Das überstehende plättchenarme Plasma wurde möglichst vollständig abgesaugt und dann vorsichtig Glutaraldehyd-Collidin (Glutaraldehyd 16%, in Collidinpuffer 0 066 mol, pH 7,3) überschichtet. Nach 10-min Inkubation bei 4° C ist das Thrombozyten-Leukozyten-Häutchen hart und kann mittels einer Öse leicht entfernt werden. Die weitere Behandlung erfolgt wie früher angegeben [26] Die Kontrastierung erfolgte mit Uranylacetat-Bleicitrat. Bei dieser Methode werden die Thrombozyten sofort nach der Blutentnahme fixiert, es bleibt daher ihre in vivo-Form (= Scheibchenform) erhalten. Verschiedene Strukturen im Mikrotubuli lassen sich mit dieser Methode besonders gut darstellen [41].

Bei der Methode B enthielt das Gemisch, in das das Blut aufgenommen wurde, keinen Glutaraldehyd, sondern nur 1 ml EDTA-Lösung und 0,5 ml 5% Dextrose. Die weitere Verarbeitung erfolgte wie bei *Methode A. Bei Anwendung dieser* Methode werden die Thrombozyten nicht sofort fixiert, sondern erst nach Aufbringen des konzentrierten Glutaraldehyds auf das Thrombozyten-Leukozyten-Häutchen. Thrombozyten, die nach dieser Methode präpariert werden, haben annähernd Kugelform, zeigen leichte Formabbildung und es kommt zu einer Separierung von Hyalomer und Granulomer Die Mikrotubuli sind kaum erkennbar hingegen ist das System der Tubuli und Bläschen sehr gut beurteilbar Bei Anwendung dieser Methode lassen sich in den Präparaten auch reichlich Leukozyten auffinden.

5. *Größenbestimmung der Thrombozyten.* Zur Größenbestimmung der Thrombozyten wurden mehrere Verfahren angewendet

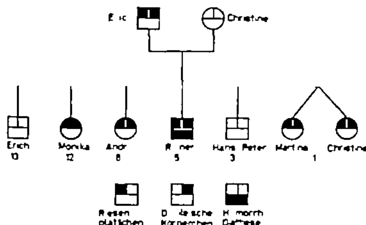


Abb. 1 Stammbaum der Familie S.

) Die Thrombozyten wurden im mit May-Grünwald-Giemsa gefärbten Ausstrichpräparat mittels eines Mesookulares ausgemessen.

b) Die Thrombozyten wurden entweder mit Methode A oder B präpariert, (Ziff. 4) und in der üblichen Weise eingebettet. Die Grösse der Thrombozyten wurde in Photographien (Endvergrösserung: 2000) von Semidünnschnitten 0,5–1 µm ausgemessen [für Details 41]. Bei vorfixierten Thrombozyten, die Plättchenform haben, wurden bei jedem Thrombozyten der Längs- und der Querdurchmesser ausgemessen, bei nicht vorfixierten, die annähernd Kugelform haben, nur ein Durchmesser. Aus den gemessenen Werten wurde die wahre Grössenverteilung der Thrombozyten mit der Methodik von Hevron [21] ermittelt. Für die kugelförmigen Thrombozyten wurde das Volumen mit der Formel 4/3 r³π und die Oberfläche mit der Formel 4 r²π berechnet.

) Zur Berechnung der mittleren Anschnittfläche in den elektronenmikroskopischen Bildern wurde ein Punktezählverfahren [39] angewendet. Je nach der elektronenmikroskopischen Vergrösserung wurde ein Punkteraster mit einem Punkteabstand von 5 oder 10 mm angelegt und die Zahl der auf einen Thrombozyten fallenden Punkte ausgezählt.

6. *Chromosomenuntersuchung* aus dem Venenblut [31]

Familie (Abb. 1)

S.E., männlich, geb. 1916, Vater der Familie, stammt aus der Schweiz. In der Aszendenz sind keine Fälle von Blutungsneigung bekannt. Der Vater des Patienten war Asthmatiker und verstarb an einem Herzleiden. Die Mutter soll an einer Krebskrankheit im hohen Alter verstorben sein. Der Patient hat eine gesunde Schwester. Er hatte als Kind häufig Nasenbluten, später nahmen sowohl Frequenz als auch Intensität des Nasenblutens ab, und in den letzten Jahren hatte der Patient fast nie mehr Nasenbluten. Nach stärkeren Traumen kommt es gelegentlich zu Hämatomen. Im übrigen hat der Patient als eine stärkere Blutungsneigung bemerkt. So verheilte im Krieg eine Splitterverletzung an der linken Hand komplikationslos. 1956 wurde eine Appendektomie ohne Blutungskomplikationen durchgeführt. Postoperativ soll es allerdings zu einer Wundeiterung gekommen sein. 1966 wurde eine Hämorrhoidenoperation, ebenfalls ohne jede Blutungskomplikationen, durchgeführt. 1968 hatte der Patient eine Hepatitis durchgemacht.

S.C., weiblich, geb. 1935, gab an, dass sie als Kind häufiger Nasenbluten gehabt habe. Ausserdem soll eine Hämatomneigung bestehen. Nach 2 Geburten kam es zu postpartalen Blutungen, wobei die Patientin einmal sogar Bluttransfusionen erhalten musste. In der Familie sind keine weiteren Fälle von Blutungsneigung bekannt. Beide Eltern der Patientin sind gesund. Von den 5 Geschwistern ist ein Bruder im 35. Lebensjahr an Magenkrebs gestorben, 4 Geschwister sind gesund.

S.E., männlich, geb. 1935 (Abb. 1) Anamnestisch keine Blutungsneigung.

S.M., weiblich, geb. 1956 (Abb. 1) leidet an häufigem Nasenbluten und Neigung zu Hämatomen.

S.A., weiblich, geb. 1960 (Abb. 1) Keine Blutungsneigung.

S.R., männlich, geb. 1963 (Abb. 1) *Das Kind hatte im 2. Lebensjahr* erstmals Nasenbluten, anfangs nur wenig und selten, später immer häufiger, schliesslich täglich. Der Blutverlust war gewöhnlich gering, gelegentlich musste das Kind aber doch tamponiert werden. 1966 kam es zu einer ungewöhnlich starken Epistaxis, so dass das Kind in ein Krankenhaus eingeliefert werden *musste und Bluttransfusionen erhielt. Wegen starken Nasenbluten* musste es seit damals noch insgesamt 5× in ein Spital aufgenommen werden. Ausserdem besteht eine Hämatomneigung nach kleinen Traumen. Blutungen anderer Art wie Hämaturie, Melaena sind nie aufgetreten. Operationen wurden bisher nicht durchgeführt.

Bei den übrigen Kindern, S.H., männlich, geb. 1965 (Abb. 1) und den im Jahre 1967 geborenen Zwillingen S.M. und S.C. (Abb. 1) ist bisher keine Blutungsneigung aufgetreten.

Die Untersuchungen der Thrombozytenfunktion und -morphologie wurden bei der Mutter (S.C.), dem Vater (S.E.) und einem Sohn (S.R.) durchgeführt.

Befunde

1. Vorkommen von Döhleschen Körperchen und Riesenplättchen

Es wurden 9 Mitglieder der Familie untersucht, und zwar die Eltern und alle Kinder (Abb. 1). Beim Vater und bei 5 der 7 Kinder konnten im *peripheren Blutausstrich* bei der May-Grünwald-Giemsa-Färbung Döhlesche Körperchen in Leukozyten und Riesenplättchen nachgewiesen werden. Die Döhleschen Körperchen zeigten die von den früheren Untersuchern angegebenen Charakteristika. Sie fanden sich vor allem in den neutrophilen *Granulozyten* und waren aber auch *in den Eosinophilen* und Monozyten vorhanden. Meistens waren sie als Halbmonde am Rand der Zelle lokalisiert, gelegentlich wurden sie als kugelige Gebilde auch im Inneren der Zelle, in der Gegend des Zellkerns gefunden. Bei der lichtmikroskopischen Betrachtung der Plättchen fielen neben ihrer Grösse auch das Vorkommen von sogenannten blauen Plättchen auf, wie sie in charakteristischer Weise für diese Krankheit beschrieben werden. Im übrigen zeigte das Blutbild keine Besonderheiten.

2. *Routinegerinnungsuntersuchungen*

Die Ergebnisse der Routinegerinnungsuntersuchungen beim Vater (S.E.), bei der Mutter (S.C.), bei einem Sohn (S.R.) und bei einer Tochter (S.A.) sind in Tabelle 1 dargestellt. Bei allen drei Patienten mit der Anomalie fanden sich eine mittelschwere Thrombozytopenie und dementsprechend ein verlängerter Heparintoleranztest, ein erniedrigter Prothrombinverbrauch und eine verminderte Retraktion. Eine plasmatische Gerinnungsstörung war nicht nachweisbar.

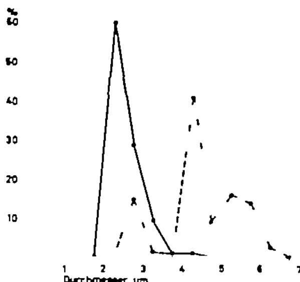


Abb. 2. Größenverteilung der Thrombozyten bei Normalen (●——●) und S.E. (●- - -●).

3. Untersuchungen an den Thrombozyten

a) Größe. Im Blutausstrich ergab sich für die Thrombozyten des Vaters ein mittlerer Durchmesser von 4,02 µm, für die des Sohnes (S.R.) von 3,26 µm, für Normalthrombozyten von 2,7 µm.

Am Schüttelpräparat nicht vorfixierter Thrombozyten [Methodik 5 b] ergab sich nach Korrektur der Werte nach der Methode von Hevoo die in Abbildung 2 dargestellte Größenverteilung der Thrombozyten. Aus diesen Werten wurden der mittlere Durchmesser, das mittlere Volumen und die mittlere Oberfläche der normalen und der Patiententhrombozyten ermittelt (Tab. II).

Beim Vergleich von Längs- und Querdurchmesser vorfixierter Thrombozyten ergab sich, dass der Quotient von Längs-Querdurchmesser bei den Patiententhrombozyten wesentlich geringer ist als bei Normalthrombozyten, was für eine plumpere Form spricht.

b) Thrombozytenfaktoren 3 und 4 (Tab. III). Die Aktivität gewaschener Patiententhrombozyten im Thromboplastinbildungstest war bezogen auf die Thrombozytenzahl normal, sowohl bei Testung intakter als auch bei durch Frieren und Tauen aufgeschlossener Thrombozyten.

Bei der Bestimmung des Thrombozytenfaktors 4 ergab sich schon, wenn die Ergebnisse auf die Thrombozytenzahl bezogen wurden, eine verminderte Freisetzung von Thrombozytenfaktor 4 wie auch eine absolute Verminderung in den aufgeschlossenen Thrombozyten. Diese Verminderung wird natürlich stärker, wenn man sie auf das Thrombozytenvolumen bezieht. Die Thrombozyten von S.R. und S.E. haben somit eine deutlich verminderte Antiheparinaktivität.

c) Th[illegible] Im Ausbreitungsbild der Patiententhrombozyten fand sich eine ausgeprägte Linksverschiebung mit Vermehrung großer Ausbreitungsformen und Riesenformen. Im Hyalomer der Riesenplättchen fallen vereinzelte Vakuolenbildungen auf.

Tabelle I. Gerinnungsbefunde

	S.E.	S.R.	S.C.	S.A.	Normal
Blutungszeit, sec	100–135	185	60–195	210	<180
Thrombozytenzahl	45 000–67 000	59 000–98 000	177 000–184 000	74 000	150 000–350 000
Retraktion [8], %	54	70	92	64	>90
Retraktion [3], %	–	44/50	57/89	–	>60/>70
Gerinnungszeit, min	6	5^{30}	8	–	5–9
Prothrombinzeit, %	85	95	95	100	70–110
Partielle Thromboplastinzeit, sec	45	50	49	–	40–55
Heparintoleranztest (0,5 E) min	5^{30}	5	5	–	3–5
(0,2 E) min	3	3^{30}	2^{45}	3^{30}	1^{30}–3
Serumprothrombin nach 1 h, %	15,5	5	22		<10
Thrombinzeit, sec	12,4	13,9	12,3		9–13
Thrombelastogramm R	13,15	14 15	16,45	12,15	10,30–15
Thrombelastogramm mE	96	122	108	89	90–150
Fibrinogen, mg%	370	295	462	–	200–500
Faktor VIII, %	75	130	100		70–180

Tabelle II. Grössenbestimmung der Thrombozyten

	Normal (n=4)	S.E.	S.R.	S.Ch.
Mittlerer Durchmesser, µm	$2,52 \pm 0,2$	4,5	4 1	2,52
Mittleres Volumen, µm³	$8,35 \pm 0,47$	48	36,5	8,35
Mittlere Oberfläche, µm	$20,1 \pm 0,8$	64	53	20
Mittlerer Längsdurchmesser, µm	$3,75 \pm 0,47$	4 7	5,3	4 1
Mittlerer Querdurchmesser, µm	$0,96 \pm 0,09$	2,0	1,8	1,23
Verh. Längs-/Querdurchmesser	3,9	2,35	3,0	3,34

d) Die Adhäsivität der Thrombozyten an silikonisiertem Glas, gemessen mit der Methode von Baldini, war im Vergleich zu Normalthrombozyten deutlich gesteigert (Tab. III).

e) Thrombozytenaggregation. Es wurde die Aggregierbarkeit der Plättchen durch ADP, Adrenalin und Kollagen in jeweils zwei Konzentrationen geprüft. In Tabelle III ist die graphisch ermittelte Initialgeschwindigkeit der Aggregation im photometrischen Test angegeben, wobei ein höherer Wert einer stärkeren Aggregation entspricht. Gegenüber einer zur gleichen Zeit getesteten Normalperson, deren plättchenreiches Plasma auf die gleiche

Tabelle III Thrombozytenfunktionsprüfungen

		S.E.	S.R.	S.C.	Normal
Ausbreitung [8]	Riesenformen u. grosse Ausbreitungsformen	32,4	39,2	–	–7,6
	kleine Ausbreitungsf.	17,6	8,0	–	32,5–78
	Übergangsformen	13,8	10,2	–	4,5–33,5
	Spindeln	36,2	42,6		–40,6
Adhäsivität an Glas [8], %		2,9	3,41		0,8–2,0
Aggregation [5] mm/min Thrombozytenzahl 80 000	ADP 50 µmol	0,9	6,2	4,2	2,6
	ADP 2 µmol	1,2	6,4	3,9	1,8
	Adrenalin 100 µmol	1,7	6,3	5,7	2,6
	Adrenalin 10 µmol	0,9	6,3	3,4	2,3
	Kollagen 1 : 10	0,4	4,2	2,0	1,1
	Kollagen 1 : 20	0,2	2,7	1,0	0,5
Serotoninaufnahme, ng/10⁸ Thrombozyten		350	400		100–200
Thrombozytenfaktor 3 (12) sec	200 000 intakt	12,4	11,6		<12
	200 000 aufgeschl.	8,8	8,2		<10
	60 000 intakt	17,8	16,6		<16
	60 000 aufgeschl.	10,6	10,4		<13
Thrombozytenfaktor 4 [12], E	10^8 intakt	0,0085	0,009		0,02–0,04
	10^8 aufgeschl.	0,0225	0,0215		0,06

Thrombozytenzahl eingestellt wurde, hatte der Sohn eine stärkere, der Vater eine leicht verringerte Initialgeschwindigkeit der Aggregation.

f) Die *Serotoninaufnahme* der Thrombozyten war erhöht. Bezieht man die Serotoninaufnahme jedoch auf die Oberfläche der Thrombozyten, ergibt sich ein normaler Wert.

4. Enzymaktivitäten und Substratkonzentrationen in den Thrombozyten

In Tabelle IV sind die Aktivitäten der Enzyme und die Konzentrationen der Substrate in den Patiententhrombozyten angegeben. Werden die Werte wie gewöhnlich für 10^8 Thrombozyten angegeben, so sind alle Enzymaktivitäten und Substratkonzentrationen erhöht, wobei allerdings das Ausmass der Erhöhung sehr unterschiedlich ist. Bezieht man die Werte jedoch auf das Thrombozytenvolumen, indem man die Werte der Patiententhrombozyten durch jenen Faktor, um den das Volumen der Patiententhrombozyten grösser ist als das von Normalthrombozyten, dividiert, so zeigt sich, dass eine Reihe von Enzymen und Substraten im Normalbereich sind, dass einige Enzyme wie die Hexokinase und die Aldolase immer noch eindeutig erhöht sind. Andere Enzyme wie die Phosphoglyceromutase, die Phosphofruktokinase, die Glutathionreduktase, sowie das ATP sind dagegen deutlich vermindert.

5. Ultrastruktur der Thrombozyten

Bei der ultrastrukturellen Untersuchung der Thrombozyten ist in erster Linie die Grössen- und Formveränderung der Thrombozyten auffallend. Man sieht in den Schnitt-

Tabelle IV Enzymaktivitäten und Substratkonzentrationen in den Thrombozyten

	S.E.	b	S.R.	b	Normal
Hexokinase	39	6,8	33,8	7,9	3,95 ± 0,5
P-Hexoseisomerase	210	36,6	203	47	81,3 ± 10,3
Fruktose-6-P Kinase	6,8	1,2	21	4,9	9,2 ± 1,6
Aldolase	33,8	5,9	30	7,0	4 1 ± 1,2
Phosphoglyceratmutase	97,5	17,0	90	21	65 ± 11,8
Glycerinaldehyd-P Dehydr	475	82,5	490	114	120 ± 29,5
Phosphoglyceratkinase	545	95	467	109	115 ± 12,2
Enolase	125	21,8	126	29	23,5 ± 6,8
Pyruvatkinase	300	52,2	394	91,5	83,2 ± 25,1
Laktatdehydrogenase	630	110	713	165	148,5 ± 24 5
Glukose-6-P-Dehydrogenase	52	9,1	37,0	8,6	10,1 ± 3,8
Glukonat-6-P Dehydrogenase	12,5	2,2	17,3	4,0	2,4 ± 0,5
Glutathionreduktase	13,9	2,4	16,3	3,8	8,5 ± 1,6
Myokinase	30	5,2	35	8,1	11,5
ATP	34,5	6,0	32,4	7,6	16,5
ADP	9 7	1 7	12,2	2,8	3,8
AMP	11,5	2,0	10,8	2,5	8,1

a) Enzymaktivitäten in IU/10^{11} Thrombozyten und Substratkonzentrationen in μmol/10^{11} Thrombozyten.

b) Enzymaktivitäten und Substratkonzentrationen in 1,74 · 10^{10} Plättchen (bei Pat. S.E.) bzw. 2,32 · 10^{10} Plättchen (bei Pat. S.R.). Das Volumen dieser Plättchenzahlen entspricht jeweils dem von 10^{11} Normalplättchen, da die Plättchen von S.E. das 5,75fache und von S.R. das 4,3fache Volumen von Normalplättchen haben.

präparaten nur selten die typischen Spindelformen, sondern meistens runde bis ovale Formen, was dafür spricht, daß die Thrombozyten weniger die Form einer Scheibe, sondern mehr eines Ellipsoids haben (Abb. 3).

Bei der quantitativen Auswertung der Zellorganellen (Tab. V) wird ersichtlich, daß die Zahl der dichten Granula und der Mitochondrien pro Thrombozyt vermehrt ist. Bezieht man diese Zahl jedoch auf die Anschnittsfläche [39], die bei diesen Thrombozyten viel größer ist, sind diese Werte annähernd normal. Auffallend war das ungewöhnlich häufige Vorkommen von schmalen, schlauchartigen dichten Granula, die entweder als Ausstülpung der normalen dichten Granula zu sehen waren oder auch keine Beziehung zu den runden dichten Granula aufwiesen (Abb. 4). Derartige Stäbchen und schlauchartige Bildungen lassen sich in großer Zahl auch in normalen Megakaryozyten finden, und es liegt daher die Vermutung nahe, daß es sich um Vorstufen der dichten Granula handelt. Diese Bildungen sind daher möglicherweise als Zeichen der Unreife der Thrombozyten zu deuten. Noch andere Zeichen der Unreife wie Ochsenaugengranula, Golgi-Felder und in einzelnen Thrombozyten sogar rauhes endoplasmatisches Retikulum lassen sich nachweisen. Das System der Tubuli und Bläschen war sehr ausgeprägt, wobei sich häufig längere zusammenhängende Schläuche nachweisen ließen. Die Anordnung dieser Schläuche erinnert an man-



Abb. 3. Vergleich von normalen und Patiententhrombozyten b Präp. nach Methode A (× 10.000)

1 µm

4

1 µm

5

Tabelle V Ultrastruktur der Thrombozyten

	S. E.		S. R.		Normal n = 10
Dichte Granula	21,14	9,9[2]	21,02[1]	12,6[2]	9,4 ± 1,6[2]
Mitochondrien	2,52	1,18[2]	2,83	1,73[2]	1,2 ± 0,28[2]
Tubuli und Bläschen	2,12		2,28		1,3 ± 0,23
Glykogen[3]	1,1		0,9		
Schmale Stäbchengranula	48%		56	33,5	4,5 ± 3,6
Dicke Stäbchengranula	0			1%	
Ochsenaugengranula	16		18[4]	10,8	0,5
Riesengranula	0		0	0	< 1%
Golgifelder[4]	2		2	1,2	< 1
Vakuolen	32[4]		36%	21,5	13 ± 4,6

Organellen pro Plättchen

Gehalt an Organellen bezogen auf die Grösse der Schnittfläche (Normal = 100%, S.E. = 13 %, S.R. = 167%)

geschätzt mit subjektiver Skala 1–4

% der Thrombozytenschnitte, die solche Organellen enthalten

chen Thrombozyten an die plättchendemarkierenden Zonen in den Megakaryozyten. In einzelnen Thrombozyten scheinen diese Tubuli den Thrombozyten direkt in einige Abschnitte zu teilen.

6. Ultrastruktur der Leukozyten

Die Döhleschen Körperchen liessen sich auch ultrastrukturell darstellen. Sie imponierten elektronenmikroskopisch als unscharf begrenzte Areale von erdichtetem Zytoplasma. Im Inneren weisen sie eine grosse Zahl von annähernd parallel verlaufenden Fibrillen auf und sind von zahlreichen Körnchen durchsetzt, die nach ihrer Farbbarkeit mit Uranylacetat und ihrer Grösse (Abb. 5) wahrscheinlich Ribosomen entsprechen dürften. Auffallend ist die nahe topographische Beziehung der Döhleschen Körperchen zu Schläuchen des rauhen endoplasmatischen Retikulums. Man trifft fast bei jedem Döhleschen Körperchen am Rand einen mehr oder weniger langen, von Ribosomen besetzten Schlauch des endoplasmatischen Retikulums an, der zuweilen das Körperchen fast einzuschliessen scheint (Abb. 6)

7. Die Chromosomenuntersuchung aus Venenblut ergab einen normalen Befund.

Abb. 4 (S.E.) Riesenthrombozyt mit unregelmässig angeordneten Mikrotubuli, Stäbchengranula (St) und Golgistrukturen (G) O = Ochsenaugengranula (× 20 000) (Methode A)

Abb. 5 (S.R.) Ausschnitt aus neutrophilem Granulozyten mit Döhleschen Körperchen. Innerhalb des Körperchens sind fibrilläre Strukturen und dazwischen granuläre Einlagerungen in einer homogenen Matrix erkennbar. Die Granula liegen in der Grössenordnung von Ribosomen (× 22 800) (Methode B)



Abb. 6. (S.R.) *Neutrophiler Granulozyt mit Einschlußkörper. Die kleinen Mitochondrien* meist gequollen. Am Rande des dichten Einschlußkörpers liegen Ribosomen-besetzte Membranen des endoplasmatischen Retikulums (→) (×12.500) (Methode B)

Diskussion

Die May-Hegglinsche Anomalie ist eine seltene Erkrankung. Insgesamt sind bisher 44 Fälle beschrieben worden, die 14 verschiedenen Familien angehören [9, 11, 16, 19, 20, 22, 23, 28, 33, 35, 36, 38, 42]. Die hier beschriebene Familie entspricht in ihrer Symptomatologie und hinsichtlich der hämatologischen Befunde der klassischen Beschreibung von Hegglin [19].

Auch der Vererbungsmodus in unserer Familie steht mit der bisherigen Annahme [9 20 28, 33 35 42] in Einklang dass die Krankheit autosomal dominant vererbt wird Die Chromosomenkultur aus dem peripheren Blut ergab einen normalen Befund LUSHER *et al* [28] fanden ebenfalls ein normales Chromosomenmuster während BUCHANAN *et al* [9] geringe chromosomale Abnormitäten fanden Es ist allerdings zu bedenken dass eine Chromosomenuntersuchung aus *dem peripheren Blut hauptsächlich Chromosomen aus den Lymphozyten* erfasst und nicht aus den Granulozyten in denen sich ja die Abnormität findet

Unsere Untersuchungen beschäftigen sich vor allem mit der Thrombozytenfunktion und morphologie bei dieser Erkrankung wobei uns vor allem interessierte ob die morphologisch abnormen Thrombozyten auch funktionell verändert sind Die zu diesem Zweck durchgeführten Thrombozytenfunktionsteste ergaben unterschiedliche Ergebnisse Bei den Thrombozytengerinnungsfaktoren wurde bezogen auf Thrombozytenzahl eine normale thromboplastische Aktivität gefunden sowohl bei Testung intakter als auch aufgeschlossener Thrombozyten Sogar wenn man das grosse Volumen dieser Thrombozyten berücksichtigt, ist die Thrombozytenfaktor 3-Aktivität noch nahezu normal Dementsprechend war auch der Prothrombinverbrauch normal, bzw nur leicht pathologisch. Damit unterscheidet sich die May Hegglinsche Anomalie wesentlich von einer anderen Riesenthrombozytopenie der Dystrophie thrombocytaire hémorrhagipare BERNARD-SOULIER» [4] bei der ein schwerer Thrombozytenfaktor 3-Mangel besteht, der einen schlechten Prothrombinverbrauch zur Folge hat [4 24 34] Allerdings fanden PETZ *et al.* [36] und LUSHER *et al* [28] auch bei der May Hegglin-Anomalie eine leichte Verminderung der thromboplastischen Aktivität der Plättchen. Die Thrombozyten-4-Aktivität war in den Patientenplättchen, schon wenn man nur auf die Thrombozytenzahl bezieht, deutlich erniedrigt. Die Retraktion war bei allen untersuchten Patienten leicht vermindert, allerdings nicht stärker als sie der Verminderung der Thrombozyten entspricht. *Wir glauben daher dass in unseren* Fällen keine Störung der Retraktionsaktivität der Thrombozyten vorliegt, während HEGGLIN *et al* [20] eine solche postulieren Die Adhäsivität der Plättchen war deutlich gesteigert, wahrscheinlich infolge der grossen Oberfläche der Plättchen. Auch die Aufnahme von markiertem Serotonin war entsprechend der Vergrösserung der

Plättchenoberfläche gesteigert. Hinsichtlich der Plättchenaggregation läßt sich nur mit Sicherheit sagen, daß sie weder bei Zusatz von ADP, Adrenalin oder Kollagen grob gestört ist. Die bekannte Schwierigkeit der Standardisierung von Thrombozytenaggregationstesten erlaubt jedoch nicht eine Aussage über geringere Unterschiede der Aggregierbarkeit dieser Plättchen. Auch NAJEAU *et al* [33] sowie LUSHER [28] fanden normale Aggregation der Patientenplättchen mit ADP und Kollagen. Im Gegensatz dazu ist die Aggregation der Plättchen bei der Bernard-Soulierschen Erkrankung eher vermindert [34]. Wenn man die Ergebnisse dieser Tests zusammenfaßt, so läßt sich sagen, daß abgesehen von einer Ausnahme (Thrombozytenfaktor 4) die Funktion dieser Riesenplättchen normal (Retraktion, Aggregation) oder sogar gesteigert ist (Adhäsivität, Ausbreitung, Serotoninaufnahme). Die vom klinischen Bild sich aufdrängende Vermutung, daß diese Thrombozyten aktiver sind als normale Thrombozyten, findet daher in den *in vitro*-Tests eine gewisse Bestätigung.

Die biochemische Untersuchung der Patiententhrombozyten lieferte [illegible] uneinheitliche Ergebnisse. Wenn, wie üblich, die Enzymaktivität auf die Thrombozytenzahl bezogen wurde, fand sich durchweg [illegible] Erhöhung der Enzymaktivitäten und der Substratkonzentrationen im Vergleich zu Normalthrombozyten. Diese Erhöhung [illegible]dings graduell sehr verschieden. Wenn man jedoch berücksichtigt, daß die Patiententhrombozyten das 4–6fache Volumen von normalen Thrombozyten haben (Tab. II) und man die Enzymwerte und [illegible] Konzentrationen der Substrate auf das Volumen der Thrombozyten bezieht, ergibt sich ein anderes Bild. Einige Enzyme (Hexokinase, Aldolase) zeigen auch dann noch eine erhöhte Aktivität, andere Enzyme wie vor allem die Phosphoglyceromutase und Glutathionreduktase hingegen eine deutliche relative Verminderung, ebenso das ATP. Diese Veränderungen waren bei beiden untersuchten Patienten in sehr ähnlicher Weise ausgeprägt. Enzymuntersuchungen an Thrombozyten mit May-Hegglinscher Anomalie wurden bisher nur von GROSS *et al* [20] durchgeführt. Unsere Ergebnisse stimmen in drei wesentlichen Punkten mit denen von GROSS *et al* überein. Erstens fanden sie ebenfalls eine generelle Zunahme der Enzymaktivitäten und Substratkonzentrationen, wenn sie auf die Thrombozytenzahl bezogen, zweitens war auch bei ihnen die Aktivitätszunahme der verschiedenen Enzyme sehr unterschiedlich und drittens konnte auch bei ihren Patienten ein deutlicher relativer Mangel an ATP

gefunden werden. Aus diesen Untersuchungen lässt sich nur mit Sicherheit sagen, dass kein isolierter schwerer Enzymdefekt vorliegt. Wieso es zu der eigentümlichen Verteilung der Enzymaktivitäten in diesen Thrombozyten kommt, ist unklar. Es läge die Vermutung nahe, dass es sich um unreife Thrombozyten handelt, die ein anderes Enzymmuster haben. Der Beweis dafür kann im Moment allerdings nicht erbracht werden, da eine isolierte Untersuchung jugendlicher Thrombozyten bisher nicht möglich war.

Bei der Morphologie der Thrombozyten ist in erster Linie die Grösse auffallend. Die Grössenverteilungskurve ergab, dass ein kleinerer Teil der Patiententhrombozyten offenbar normal gross ist, während der grössere Teil Makrothrombozyten sind, mit einem Häufigkeitsmaximum zwischen 4 und 4,5 µm Durchmesser. Eine ähnliche doppelgipfelige Verteilungskurve fand auch HEGGLIN [20]. Aus dem Vergleich von Längs- und Querdurchmesser im elektronenmikroskopischen Schnitt konnte auch wahrscheinlich gemacht werden, dass die Thrombozyten dieser Patienten nicht nur grösser, sondern auch dicker und plumper als normale sind.

Die ultrastrukturelle Untersuchung ergab einen erhöhten Gehalt der normalen Zellorganellen, wie der α-Granula und der Mitochondrien pro Thrombozyt. Bezogen auf die Anschnittfläche ist die Zahl der Organellen jedoch normal. Auffallend ist das häufige Vorkommen von ungewöhnlichen Formen, wie Stäbchengranula oder schlauchartigen Granula sowie Ochsenaugengranula und Stücken des rauhen endoplasmatischen Retikulums. Diese Veränderungen sind aber keineswegs spezifisch [26], sondern lassen nur auf eine Unreife dieser Thrombozyten schliessen. Auch HEGGLIN *et al.* [20] fanden in ihrem Fall eine hohe Zahl von Stäbchengranula. LUSHER [28] fand hingegen eine normale Struktur der Thrombozyten. Im Hinblick auf die Pathogenese bedeutsam konnte der Nachweis schlauchartiger Bildungen, ähnlich der plättchendemarkierenden Zonen der Megakaryozyten, in den Makrothrombozyten sein. Auch NAJEAN *et al.* [33] beschreiben derartige Bildungen. Dies konnte die Folge der von HEGGLIN [19, 20] an den Megakaryozyten beschriebenen Demarkierungsstörung der Plättchen sein.

Die Döhleschen Körperchen entsprechen in ihrer Ultrastruktur den Beschreibungen früherer Untersucher [23, 28, 33]. Wir möchten jedoch noch hinzufügen, dass uns die nahe topographische Beziehung von Schläuchen des rauhen endoplasmatischen Retikulums zu den

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Adresse der Autoren: Drs. K. Lechner, K. Moser, I. Medizinische Klinik der Universität, Spitalgasse 23, A-1090 Wien; Prof. Dr. L. Stockinger, Hist. embryolog. Inst. d. Universität Wien (Wien, Österreich); PD Dr. K. Breddin, Dr. E. Wenzel, I. Med. Klinik der Universität Frankfurt, *Frankfurt a. Main* (Deutschland)

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Department of Clinical Therapeutics, Athens University Medical School (Director: Prof. B. Malamos), Radioisotope Department (Director: Dr. E. Gyftaki), Alexandra Hospital and Blood Research Laboratory of the Hellenic Red Cross (Director: Dr. M. Constantoulakis), Athens

Urinary and Faecal Iron Excretion in Thalassaemia Syndromes

B. Malamos, M. Constantoulakis, E. Gyftaki, M. Kesse Elias and O. Augoustaki

Although it is accepted that normaly only low amounts of iron escape from the body with urine and faeces, there are several clinical conditions where the excretion may be elevated. Radioiron has been used by several investigators for this study in animals and in man. In animals the iron excretion was studied by Hahn *et al.* [6], Austoni and Greenberg [1] and by Copp and Greenberg [2]. In man, studies of short and long term excretion of radioiron were also carried out. Thus Dubach *et al.* [4] explored the problem of iron excretion in faeces and sweat in different haematological conditions. They found two patients with congenital haemolytic anaemias who were losing higher amounts of iron than normal persons. Green *et al.* [5] have attempted to document obligatory iron losses in adult normal males and iron loaded subjects using a variety of isotopic and chemical methods. The total loss of iron in iron loaded subjects was higher than in normals. The excretion of iron in thalassaemia has not been studied, with the exception of one case [13], although haemolysis takes place and there is a significant accumulation and disturbance of iron metabolism.

In the present work the loss of i.v. administered iron was studied in urine and faeces of patients with heterozygous and homozygous thalassaemia, sickle cell thalassaemia and in normal subjects.

Material and Methods

After i.v. administration of ^{59}Fe the excretion of the radioisotope was studied in the urine and the faeces in 17 patients with various types of thalassaemia and in 4 non-anaemic patients.

A sample of the patients own plasma was incubated *in vitro* for / h with about 10 µc of ^{59}Fe in adults and 4 µc in children below the 14th year of age; the mixture was injected i.v. The administered ^{59}Fe is considered as completely bound to transferrin since its specific activity was high (1 µc/0.15 µg) and the volume of the available plasma always large.

The collection of the urine and the faeces started after the injection of ^{59}Fe. Twenty four-hours urine specimens were collected for at least 5 days and in some patients for 10 days. In 6 patients urine was collected separately at 3 h intervals for the first 24 h.

Iron concentration was determined in each sample chemically with a modification of the method for blood of Kok and Wild [9]. Radioactivity of the samples was measured in a well type scintillation counter (Nuclear Chicago No. 132).

Since the volume, which can be measured in the well counter is limited and because the radioactivity in the samples was low 100 ml of urine were placed in dialysis bags and left in front of an air stream to be evaporated and be reduced to final volume of 4 ml.

Faeces were collected separately every 24 h for 5 days and in some patients for 10 days. The faeces of each day were diluted with HCl and homogenized. Then they were dehydrated by heat. The dry remaining was weighed and a sample of known weight was assayed for radioactivity. The results of radioassay are expressed as the percentage of administered radioactivity which was excreted in the whole amount of urine or faeces per day.

Detection of transferrin in the concentrated urine of 3 patients was carried out using immunodiffusion with specific antitransferrin serum (obtained from Hyland Laboratories, Los Angeles, CA). Transferrin in concentrated urine was quantitated by the radial diffusion technique (Immunoplates obtained from Hyland Laboratories).

Results

The haematologic data of the patients studied are shown in table I.

Urine. The results of ^{59}Fe urine excretion after i.v. administration are expressed as first day excretion and as total amount from day 2–5 and from day 2–10 separately for each patient. The mean values for each group are also calculated (table II and fig. 1). The ^{59}Fe excretion during the first 24 h is quite high in all the groups studied with a wide range which in general corresponds to the levels of excretion of the subsequent days.

In order to find when the maximum ^{59}Fe excretion occurred during the first 24 h, radioactivity was counted in urine collected at 3 hourly intervals (table III and fig. 2). In all the patients irrespective of disease, the maximum of the excreted radioactivity occurs during the first 3 h after the i.v. administration of transferrin bound ^{59}Fe.

During the subsequent days the control patients excreted per day

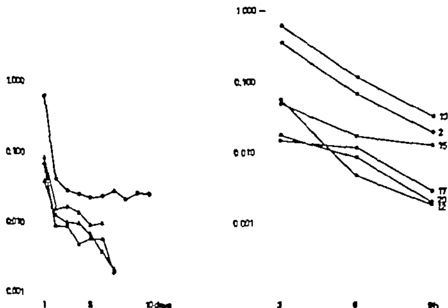


Fig. 1 Urinary ^{59}Fe loss (mean value per day % of the administered dose) ○ Thalassaemia ● thalassaemia *trait*; ▲ S/Th △ controls.

Fig. 2. Urinary ^{59}Fe loss 3 h intervals during the first day (% of the administered dose). The numbers of the curves correspond to the case numbers of the tables I, II.

very low amounts of ^{59}Fe, which ranged from 0 002 to 0.013 % of the injected dose (fig 1) Similar results were obtained in heterozygous thalassaemia. In microdrepanocytic anaemia (thalassaemia-haemoglobin S disease) the excretion is somewhat higher than in the above groups. The excretion in homozygous thalassaemia is significantly higher and more or less stable during all the days of observation ranging from 0 022 to 0 042 % per day (fig 1)

In 5 patients the concentrated urine used for counting the radioactivity was tested for the presence of transferrin, using the immunodiffusion technique with a specific antitransferrin serum. All samples tested gave a well defined precipitin line, indicating the presence of transferrin.

The quantitative estimation of transferrin was carried out in the urine of 4 patients. One to three samples of urine of different days from each patient were used. In case No 8 the amount of transferrin

Table I. Haematological data

Case No.	Age years	Sex	Hb g%	HCT %	Hb electroph.	Serum Iron µg%	Iron excretion in urine mg/24 h	Splenect.	Transfus.
Thalassaemia									
1	12	M	8.3	31	F(30%)	222	1.28	yes	frequent
2	28	M	6.1	21	A_1A_2F(40%)	131	0	no	rare
3	21	F	7.8	29	A_1A_2F(20%)	76	0	no	frequent
4	7	F	5.7	19	A_1A_2F(50%)	225	0.67	no	frequent
5	14	M	7 7	28	F(90%)	280	0.98	yes	frequent
6	32	F	7.2	23.5	A_2F(80%)	120	–	no	no
7	27	M	8.6	30	A_1A_2F(60%)	160	0	yes	rare
8	20	F	10.5	34	A_1A_2F(60%)	115	0	no	frequent
9	9	M	6.3	24	F(35%)	160	0	no	frequent
10	8	F	8.7	29	A_1A_2F	218	0	no	rare
11	12	M	8.3	28	F(75%)	240	0.40	yes	frequent
Thalassaemia trait									
12	24	F	11 7	36	A_1A_2F(2%)	127	0	no	no
13	34	F	11 4	39	A_1A_2F(2%)	127	0	no	no
14	39	F	12.5	40.5	A_1A_2F(4.7%)	–	0	no	no
15	50	F	12.5	43	A_1A_2F(traces)	124	traces	no	no
S/Th.									
16	25	F	7 7	25	A_1F,S	210	–	no	no
17	21	F	7.2	28	A_1F,S(F=5%)	102	traces	no	no
Controls									
18	48	M	–	–	normal	103	0	no	no
19	42	M	13.2	35	normal	79	traces	no	no
20	53	M	16.4	49	normal	158	0	no	no
21	22	F	13 4	43	normal	–	0	no	no

was approximately 0.25 mg/100 ml urine and in cases No. 9 and 10 0 1 mg/100 ml of urine. Because the amount of transferrin in the urine was very small and the method used is accurate only (for much higher concentrations 10 mg ‰) the results cannot be considered dependable except in the sense that they demonstrate the presence of transferrin.

Faeces The excretion of ^{59}Fe in the faeces during the first 5 days varies among the different groups studied but to a lesser extent as compared with urine (table II) It is interesting that the faecal excretion is inversed as compared with urine, except in the group of micro-

Table II ^{59}F loss (percentage of the administered dose)

Case No.	Urine 1st day	Total amount from day 2 5	Total amount from day 2 10	Faeces Total amount for the first 5 days	Total amount from day 6–10
Thalassaemia					
1	1.644	0.294	0.522	0.077[2]	0.148
2	0.450	0.021	0.034	0.065	0.220
3	0.127	0.027	0.040	0.035	0.040
4	0.292	0.103	0.201	0.111	0.100
5	2.697	0.363	0.819	0.377	0.260
6	0.077	0.082	–	0.034	–
7	0.035	0.0[illegible]9	–	0.066	–
8	0.087	0.030	–	0.060	–
9	0.639	0.045	–	0.074	–
10	0.855	–	–	0.037	–
11	0.091	–	–	0.124	–
	0.637[1]	0.114	0.523	0.098[1]	0.154
Thalassaemia trait					
12	0.064	0.018		0.069	0.097
13	0.099	0.027	–	–	–
14	0.015	0.025	–	0.103	
15	0.108	0.048		0.291	–
	0.072[1]	0.030	–	0.162[1]	–
S/Th					
16	0.068	0.072	0.105	0.045	0.070
17	0.032	–		0.155	–
	0.060[1]		–	0.100[1]	
Normal					
18	0.025	0.024		0.125	0.048[2]
19	0.016	0.064	–	0.083	0.151
20	0.036	0.013		0.121	
21	0.103	0.056		0.192	–
	0.045[1]	0.040		0.130	0.100

Mean value.
Faecal collection up to 8th day

drepanocytic anaemia in which the iron found was low So in homozygous thalassaemia the mean faecal excretion per 5 days is 0 098 % and in controls 0.130 %.

Table III. Urinary ^{59}Fe loss in 3 h intervals during the first day (percentage of administered dose)

Case No.	Hours after ^{59}Fe administration							
	3	6	9	12	15	18	21	24
4[1]	0.561	0.069	0.021	0.003	0.001	0.002	0.001	0.001
15[2]	0.051	0.018	0.014	0.009	0.005	0.002	0.005	0.004

Homozygous thalassaemia.
Heterozygous thalassaemia.

The faecal excretion during the first day was very low and there after became much higher with wide daily variations during the 5- to 10-day period of collection. The wide daily variation may be accounted for by the different volumes of faeces passed by the patients. However there was no significant change of the ^{59}Fe excreted between the first and second 5-day period in the patients tested (table II)

Discussion

All our cases showed maximum urinary excretion of radioiron during the first 24 h and when collection in 6 patients was performed at 3 h intervals (fig 2) the maximum excretion occurred during the first 3 h. The early urinary iron excretion, could be in part related to the presence of a considerable amount of diffusible iron in the plasma for a short interval [6] However in the present work, the administered ^{59}Fe was of high specific activity and was incubated with large amounts of plasma, thus bound to transferrin. In addition in case 11 ^{59}Fe was incubated *in vitro* with the plasma of the patient's mother Further similar increased ^{59}Fe excretion was noted in normal controls who have a high percentage of unbound transferrin in their plasma. Therefore the early ^{59}Fe loss can be attributed to a normal mechanism of transferrin-Fe excretion occurring when iron circulates bound with transferrin before it leaves the plasma going to the bone marrow and the iron stores. In all urine samples tested, transferrin was detected and further it was too early to find radioactive haemoglobin iron from haemolyzed red cells. In favour of this assumption are the results of

DAGO *et al* [3] and TRAEGER *et al* [15] who found increased sideruria in patients with proteinuria and siderophilinuria. Contrary to the first hours most of the urinary ^{59}Fe in the subsequent days is probably due to exfoliated epithelial cells and erythrocytes as suggested by others [3 5]

The highest ^{59}Fe excretion, both of the first 24 h and of the subsequent days, was observed in thalassaemic patients. This can be explained in addition to the above factors by the recirculation of ^{59}Fe [11 12] and by the severe degree of haemosiderosis and increased saturation of transferrin which causes non-specific unloading of iron to various tissues [8] In support of this possibility are our findings that thalassaemic patients with high serum iron levels, excrete more iron than patients with low levels (with one exception No 4) The same applies to patients with clinical haemosiderosis and numerous transfusions, in whom the excretion is much higher [14] SEARS *et al.* [13] also state that in haemolytic anaemias the significantly increased urinary iron excretion is due to haemosiderinuria and exfoliation of iron loaded epithelial urinary tract cells. They found in addition, in the one thalassaemic patient studied, that the daily ^{59}Fe urinary excretion was 3 times higher than normal. In our study this difference, as a mean is more pronounced, being about 10 times above normal.

Among the groups studied the urinary excretion, both during the first 24 h and the 5- to 10-day period, varies between the lowest in control patients and the highest in thalassaemics. The heterozygote thalassaemics excreted similar amounts as the controls, while the double heterozygotes for thalassaemia haemoglobin S disease showed slightly higher amounts in the urine In the above groups no significant increase in serum iron levels was found, which could partially account for the higher and continued excretion. It is possible that in all groups, including thalassaemia, intravascular haemolysis may have been also responsible. This view is reinforced by the higher excretion of iron found in haptoglobin depleted patients [13]

The amount of excreted radioiron in faeces was low and comparable to the previously reported values [7 4] If one accepts that the faecal iron comes from the sloughing of epithelial cells and intestinal blood loss, then the very low radioactivity of the first day is not unexplained. The faecal radioactivity of the first 5-day period did not vary significantly among the groups studied, although there is a tendency to be reverse in comparison with the urinary excretion. This could be due

to either less ^{59}Fe uptake by the already loaded epithelial cells in thalassaemics, in contrast to the normals and heterozygotes, or to the excretion by the urine of the available for diffusion quantity

It is interesting that increased levels of radioactivity were obtained during the second 5-day period in thalassaemia but not in controls. Green et al. [5] reported that in normal subjects the faecal ^{59}Fe activity in the stools drops after day 5–8 This finding may suggest that two mechanisms are responsible for the late increase of faecal radioactivity in our thalassaemic patients (a) sloughing of the initially labelled epithelial cells whose turnover rate according to Lipkin et al. [10] was calculated to be between one and 4 days and (b) biliary ^{59}Fe especially for the sustained radioactivity considering that most of the patients investigated had variable degrees of dyshaemopoiesis (ineffective erythropoiesis, low red cell iron uptake)

That the faecal radioactivity was lower in thalassaemics initially does not oppose the assumption that the total iron excretion in the stool is significantly higher in haemosiderotic patients than in normal controls.

Summary

Determination of the percentage of radioiron excretion in faeces and urine has been made, after i.v. ^{59}F administration, in thalassaemic patients, carriers of thalassaemia trait, patients with sickle cell thalassaemia and normal subjects. All the cases studied showed maximum urinary excretion of radioiron during the first 24 h. The highest excretion both of the first 24 h and of the subsequent days was observed in thalassaemia patients. The excretion of ^{59}Fe in the faeces during the first 3 days varies among the different groups studied and it is inversed as compared with urine being higher in thalassaemia trait and in normal subjects. The interpretation of the results is discussed.

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Authors' addresses: Prof. Dr. B. Malamos, Dr. E. Gyftaki and Dr. M. Kesse-Elias, Department of Clinical Therapeutics, University of Athens, Alexandra Hospital, Athens; Dr. M. Constantoulakis, and Dr. O. Aloocutaki, Blood Research Laboratory, Hellenic Red Cross, Athens (Greece)

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Department of Medicine, St. Elizabeth Hospital, Boston, and Tufts University School of Medicine

Megakaryocytes and Platelets in Central Venous and Arterial Blood[1]

Alice Kallinikos-Maniatis

The observation that megakaryocytes inhabit the capillaries of the lung dates back to 1893 [1] and since then the question of their origin has been a matter of controversy [2 3] The presence of megakaryocytes in the blood was originally thought to be associated only with pathologic conditions in which the bone marrow was under strain (i e myeloid metaplasia, chronic myelogenous leukemia sepsis [4]) as well as with malignancies [5] More recently megakaryocytes have been recognized as normal inhabitants of the blood since they are always found although in small numbers in the blood of normal individuals [6 7 8]

It is the opinion of most investigators at the present that circulating megakaryocytes are produced in the bone marrow and are trapped in the capillaries of the lungs where they release platelets [3 7 8] If this is true a significantly larger number of megakaryocytes should be present in the venous as compared to the arterial blood.

The following study was undertaken to determine the difference in numbers of megakaryocytes as well as platelets between the central venous and central arterial blood in subjects undergoing cardiac catheterization who were normal from the hematologic standpoint Previous studies have been done on patients undergoing thoracotomy for pulmonary diseases [8, 9]

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Materials and Methods

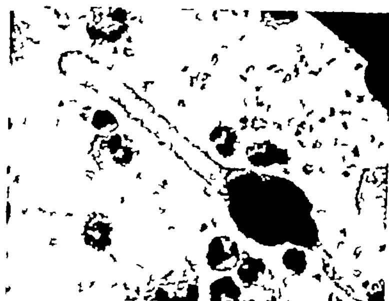
Ten human subjects, 7 females and 3 males ranging in ge from 15 to 62 years were included in this study The abov subjects were undergoing diagnostic cardiac catheterization; 7 of them were found to ha cardiac abnormalities, 2 were considered normal and 1 was thought to ha cardiac tumor but this w not proven on exploration. Patients were sedated with Vistaril 100 mg i.m., prior to the catheterization. A total of 20 ml of blood was obtained from each patient, prior to injection of any radio-opaque media, 10 ml from the pulmonary artery (or the high right trium, if left to right shunt was present) and 10 ml from the aorta. The blood was collected in plastic syringes, through Cournand polyethylene catheter for the enous blood and Kifa or TSP catheter for the arterial. The catheters were flushed with heparinized 5 % dextrose in w er solution (20 mg of heparin in 1,000 ml 5 % D/W) prior to sampling The blood was immediately placed in buffered formalin-polyvinylpyrrolidone (PVP) fixath and the saponin-hemolysis leukoconcentration technique of HERBETVAL [10] as modified by KAUFMAN [7] was followed. By this technique all the nucleated blood cells are concentrated on millipore filters and the megakaryocytes either with or without cytoplasm can be easily recognized and counted by scanning of the filters under low power of the light microscope Megakaryocytes with cytoplasm appear elongated presumably because of their passage through narrow caches (fig 1). Only cells with intact nuclei were included in the counts.

Platelet counts were performed according to the direct method of BRECHER and CRONKITE [11]. Eight simultaneous counts were done on each sample; this has been shown to reduce the error of the mean t 4 % [12]

Results

In all 10 patients studied megakaryocytes were present in both the central venous and central arterial blood (table I) The numbers in the central venous blood varied from 3–32 cells per 10 ml of blood, whereas only 1–4 cells/10 ml were found in central arterial blood. Cells with cytoplasm varying in numbers from 2–19/10 ml with an average of 10 3 were present in the central venous blood of all 10 patients, while in the central arterial blood of only 3 patients were cells with cytoplasm found (1 or 2/10 ml)

Platelet numbers were determined in 9 patients, 8 of whom showed larger numbers in the central arterial blood with a difference between arterial and venous blood ranging from 30 000–80 000/mm with a mean of 43,500 this difference is statistically significant ($p = 0\ 001$) Only one patient (M. P) showed approximately equal numbers in both samples. The morphological appearance of the megakaryocytes was similar to that described by KAUFMAN *et al* [7] with a predominance of elongated or paddle-shaped cells (fig 1)



Megakaryocyte with elongated cytoplasm in the central venous blood (1,000

Discussion

I regoing experimental data confirm the observation that c k cytes are found in the circulating blood of normal subjects. I obtained in this study are slightly different from those re KAUFMAN *et al* [7] The average number of megakaryo- r tral venous blood was 1 78/ml (range 0.3–3 ?) as compared + c lls ml found by KAUFMAN This difference can be ex t l t the fact that the above authors included in their counts l t gments while only intact nuclei were counted in the pres- nt tud MELAMED *et al* [8] reported much larger numbers of mega kar t in antecubital venous blood (range 1–100/ml) This n pre t y due to the use of a technique which is less traumatic to th n g k ryocytes.

II r us blood was found to contain 6 times as many mega- kary t a the arterial blood and approximately 25 times more megakar ytes with cytoplasm. KAUFMAN *et al* [7] in their study found milar differences but their data are inadequate since arterial sampl w r btained from two subjects. The authors do not specify

Table I. Numbers of megakaryocytes and platelets in the central arterial and venous blood of 10 subjects

Patient	Diagnosis	Megakaryocytes 10 ml				Platelets mm³		
		Venous blood		Arterial blood		Arterial blood	Venous blood	Difference arterial-venous
		Total No.	With cytoplasm	Total No.	With cytoplasm			
C.C.	Mitral stenosis	22	19	2	0			–
J.B.	Normal	8	4	3	0	4 3 000	423 400	31,600
A.B.	Atrial septal defect	32	17	4	0	[illegible]07,500	177,500	30 000
B.L.	Aortic stenosis and insufficiency	[illegible]0	7	5	0	25[illegible],000	217 000	38,000
R.J.		19	9	4	1	232,[illegible]00	162,500	70,000
A.K.	Aortic stenosis	13	10	4	2	203,000	125 000	80 000
D.C.	Coronary artery disease	16	11	2	0	152,[illegible]00	120 000	32,500
M.P.	Normal	15	9	4	1	2[illegible]0 000	233 000	5,000
R.N.	Mitral stenosis	3	2	1	0	227 [illegible]00	162,[illegible]00	63,000
O.R.	Mitral stenosis	30	15	3	0	282,[illegible]00	232,[illegible]00	[illegible]0,000
	Average	17.8	10.3	3	0.4			43,500

whether these were central or peripheral blood samples in the latter case the difference could be attributed to destruction of megakaryocytes in the circulation and not to trapping in the lungs In the study by SCHIRMER and KOIVUNIEMI [9] it is stated that megakaryocytes were definitely more frequent in pulmonary arterial blood than in pulmonary venous blood but the actual numbers of megakaryocytes are expressed per sample of blood, the volume of which is not specified. Furthermo the ubjects used were patients with pulmonary diseases (mostl malignancies) undergoing thoracotomy Both the disease [5] as well a the operation could hav influenced the results [13]

As to the question of platelet release in the lungs the data indicate a definite enrichment of platelets in the blood leaving the lungs Simultaneously obtained red c ll counts indicated th t this was not due to

hemoconcentration. Tocantins [14] using his method for counting platelets reported similar differences between arterial and venous blood. This platelet gradient across the pulmonary vascular bed however cannot be accounted for by platelet production from the circulating megakaryocytes alone. It has been estimated that the number of platelets produced by one megakaryocyte is ∼4 000. The megakaryocytes therefore that arrive to the lungs could only account for a small fraction of the observed platelet number difference between arterial and venous blood. One could assume that the lungs act as a pool of platelets and that the experimental conditions (i. e. cardiac catheterization) disturb the existing steady state causing a release of platelets into the arterial blood. Aster however in his study was unable to demonstrate any significant pool of platelets in the lung [15].

It is finally possible that the adhesiveness of platelets in the venous blood is greater than it is in the oxygenated arterial blood and this may lead to clumping of platelets and a falsely low value. Although clumps were not observed in the counting chamber this possibility is under further investigation at the present time.

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Summary

Megakaryocyte and platelet numbers were determined in blood samples obtained during cardiac catheterization from the pulmonary artery and the aorta of 10 hematologically normal subjects. Significantly larger numbers of megakaryocytes were found in the pulmonary artery as compared to the aorta; platelet numbers on the opposite were higher in the arterial than in the venous blood. It is concluded that passage of the megakaryocytes through the lung capillaries results in fragmentation of their cytoplasm and release of platelets, while the nuclei are mostly retained in the capillaries.

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Author's address: Dr Alice Kaufman-Mandl [illegible], Blood Research Laboratory New England Medical Center Hospitals, 171 Harrison Avenue, Boston, Mass. 02111 (USA)

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Medizinische Universitätsklinik, Sektion für Hämatologie und klinische Immunologie, Göttingen

Panmyelophthise und Virushepatitis

Experimentelle Untersuchungen mit dem Mäusehepatitis-Virus MHV

W. Hunstein, E. Perings, B. Eggeling und N. Uhl

Im Gefolge der Virushepatitis des Menschen können sich in sehr seltenen Fällen Panmyelophthisen entwickeln. Solche Komplikationen bei einer Infektion mit hepatotropen Viren erwecken doppeltes Interesse: einmal werden sie als Seltenheit registriert und zumeist publiziert, zum anderen steht eine Erklärung für das Phänomen der Koinzidenz dieser verschiedenartigen Erkrankungen aus.

In der Absicht, einen Beitrag zur Klärung dieser Frage zu liefern, unternahmen wir tierexperimentelle Untersuchungen mit dem Mäusehepatitis-Virus MHV_3 [10].

Material und Methode

Als Versuchstiere dienten 96 infantile männliche und weibliche Mäuse vom Stamm CF mit einem Gewicht von 10–12 g. Dieser Stamm ist im Gegensatz zu anderen Mäusestämmen nicht gegen das MHV_3-Virus resistent. Das Virus-Lyophilisat (MHV) bezogen wir von der Type-Culture-Collection Washington, D.C.

Da es sich bei unseren Experimenten um orientierende Untersuchungen handelte, verzichteten wir auf die bei Virusexperimenten üblichen Titerbestimmungen in den von uns hergestellten Injektionslösungen. Wir gingen von einer Verdünnungsreihe aus. Eine Stammlösung von 1 ml wurde gemäß dem Ergebnis eigener Vorversuche, mit 0,9% NaCl-Lösung zu 1:10, 1:20, 1:50, 1:100 und 1:200 verdünnt. Intraperitoneal injiziert wurden 0,01 ml der jeweiligen Verdünnung pro g Körpergewicht bei 5 Gruppen von je 18 Mäusen. 6 Tiere dienten als Kontrollgruppe. 72 Stunden nach Injektion dekapitierten wir aus den Gruppen A und B 6 Mäuse, und zwar diejenigen, die sich im schlechtesten Allgemeinzustand befanden. Die rapide Verschlechterung des Zustandes der Mäuse in Gruppe A und B (Tab. 1) veranlaßte uns zur Tötung aller Tiere dieser Gruppe nach weiteren 6 Stunden. Die Tiere der Gruppen C, D und E töteten wir wie vorgesehen nach 84, 132 bzw. 180 h. Tötung der Kontrolltiere nach 3 Tagen.

Sternum und Oberschenkel sowie Leber, Lun[illegible] Niere und Milz wurden in For[illegible]mol fixiert und in üblicher Weise histologisch [illegible]. Ein Teil des Gewebes wurde in Metacryla[illegible] eingebettet.

Ergeb[illegible]

1. Verhalten der Tiere

Zwischen dem 2. und 3. Tag nach V[illegible]chsbeginn traten allgemeine Krankheitszeichen auf wie Fress- und Bewegungsunlust, struppiges Fell und ante finem hastige Atmung und [illegible]stammengekrümmte Haltung. Diese Symptome beobachteten wir in allen Versuchsgruppen häufiger nach Gabe der höheren Viruskonzentrationen. Ebenso befanden sich in allen Gruppen Tiere, die aus[illegible]lich keine Anzeichen einer Erkrankung hatten, besonders in den Gruppen mit 1:100 und 1:200 der Stammlösung.

2. Histologische Knochenmarkbefunde

Gruppe A (1:10 MHV$_{3}$, 72 bzw. 78 h *p. i.* getötet). Bei allen Tieren fanden sich ausgedehnte Nekrosen (Abb. 1), zumeist Kolliquationsnekrosen. Zwar war in der Regel das Mark in ganzer Ausdehnung befallen, doch liessen sich herdförmige Bezirke mit besonders ausgeprägter Zellzerstörung und Ansammlung von Ödemflüssigkeit nachweisen. Solche Areale erinnerten an metastatisch-septische «Mikroabszesse». Reparative Vorgänge waren nur bei wenigen Tieren nachweisbar.

Gruppe B (1:20 MHV$_{3}$, Tötung nach 72 bzw. 78 h). Auch in dieser Gruppe zeigten – bis auf eine Ausnahme – alle Tiere Nekrosen in Sternum und Femur. Bei einem Tier bestand im Femur eine ausgedehnte Markaplasie, gleichzeitig waren grossflächige Nekrosen im Sternum entwickelt. Bei 2 weiteren Tieren fanden sich grosse aplastische Bezirke neben kleineren Nekrosearealen. Bei einem Teil der Tiere war es bereits zu lebhaften reparativen Vorgängen mit Phagozytose und retikulärer Reaktion gekommen. Auch bestand eine Zunahme von Proerythroblasten und Promyelozyten unmittelbar neben noch nekrotischen Arealen.

Gruppe C_1–C_4 (1:50 MHV$_{3}$, Tötung nach 84 h). 2 Tiere zeigten

Tabelle I Histologische Befunde in Beziehung zu Versuchsdauer und Virusverdünnung (15 vorzeitig gestorbene Tiere blieben unberücksichtigt)

Virus-verdünnung	Zeit in Stunden 72	78	84	132	180
1:200			× ○	□□	□□
			× × ○	□□□	□□
1:100			× □□	□□□	□□□
			× □□	□□□	□□□
1:50			× □	○□	□□□
			× ○□	○○□	□□
1:20	× ○	○			
	× ○	× ○○			
		× ○○□			
1:10	× ×	○○			
		○○			
		× ○○			

× Ausgedehnte Nekrosen, ○ diskrete Nekrosen, □ ohne Befund

massive Nekrosen; bei 2 Tieren war das Mark normal. Bei einem Tier fanden sich nur diskrete Einzelzellnekrosen.

In Gruppe C_6–C_{11} (Tötung nach 132 h) wiesen 3 Tiere Einzelzellnekrosen auf. Häufiger fanden sich mit Kernresten überladene Phagen (Abb. 2). Noch immer bestand eine Zunahme der Mitoserate, doch war die «Linksverschiebung» nicht mehr so auffällig. 2 Tiere blieben ohne Befund.

Tiere der Gruppe C_1–C_{18} (Tötung nach 180 h) zeigten keine Veränderungen mehr.

In der Gruppe D–D_6 (1:100 MHV_3, Tötung nach 84 h) zeigten 2 Tiere frische, ausgesprochen kleinherdige Nekrosen (Abb. 3). Die nach *132 bzw. 180 h getöteten Tiere dieser Gruppe (D_7–D_{12}) boten einen* praktisch normalen Markbefund. Vereinzelt waren Schaumzellen und phagozytierende Retikulumzellen als Residuen eines vorangegangenen *Markschadens auffindbar.*

Die *Kontrolltiere* zeigten einen normalen Markbefund.

Ähnliches gilt für die *Gruppe E.* Auf der Tabelle I sind die histologischen Befunde in Korrelation zu Versuchsdauer und zur injizierten Virusverdünnung aufgetragen. Die Tabelle zeigt, dass mit Zunahme

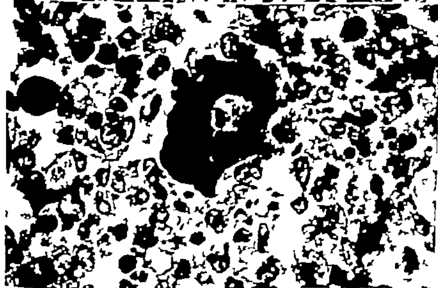


Abb. 1. Ausgedehnte diffuse, herdförmig betonte Knochenmarknekrosen mit zahlreichen kugeligen pyknomorphen Kernresten. Kleine Ödembezirke. Einzelne Schaumzellen. Oberschenkel (Masson-Goldner, 420×)

Abb. 2. Abräumzelle mit zahllosen Kernresten im Zytoplasma. Der Zellkern tritt als Aussparung hervor. Angrenzend große promyelozytäre Elemente. Außerdem noch nicht phagozytiertes Kernmaterial überall im Bild verteilt. Links außen kleinere phagozytierende Zelle. Sternum (Masson-Goldner, 670×)

[11 16] Temporäre Verminderungen eines oder mehrerer hämopoetischer Zellsysteme sind bei verschiedenen Viren bekannt, z.B. beim Mumps Virus [9] dem Röteln Virus [27] und beim Denguefieber [5] FIERRO und BERKOVICH [9a] haben kürzlich nach Infektion mit Coxsackie B_4 Virus bei Mäusen temporäre Aplasien der Erythropoese erzeugt

Unsere Befunde zeigen nun erstmals, dass für die hämatologischen Veränderungen nach MHV_3 Virus massive Knochenmarknekrosen verantwortlich sind. Wir fanden in allen Tiergruppen Nekrosen unterschiedlichen Alters. Sie reichen vom Bild der frischen Entparenchymisierung mit Ödemseen (Abb 1) bis zu kleinen herdförmigen Gruppennekrosen hämopoetischer Zellen (Abb 3) Stets waren die Veränderungen an verschiedenen Stellen der untersuchten Markabschnitte anzutreffen Die nekrotischen Zellreste wurden im Ablauf einer Reparationsphase von retikulären Abräumzellen aufgenommen und eliminiert. Häufig liessen sich in späten Stadien mit zahllosen Kernresten angefüllte Speicherzellen nachweisen (Abb 2) Die angrenzenden, intakt gebliebenen Gewebsabschnitte zeigten Regenerationsansätze mit Vermehrung myelopoetischer Vorstufen und gehäuft Kernteilungsfiguren.

Nur in einem Fall war es nicht zur reparativen Wiederbesiedlung des zerstörten Knochenmarks gekommen. Es hatte sich vielmehr eine Hypoplasie des blutbildenden Gewebes entwickelt. Bei diesem Tier fand sich ein Bild, wie es bei der Markaplasie nach Virushepatitis des Menschen beschrieben worden ist Zwei weitere Tiere zeigten ein hypoplastisches Mark, aber keine vollständige Phthise

Die im Knochenmark nachgewiesenen Nekrosen sind das Analogon zu Veränderungen die in Milz und anderen lymphatischen Geweben schon durch HIRANO und RUEBNER [12, 13] festgestellt worden waren Die Knochenmarkbefunde blieben bisher unerkannt was sich daraus erklärt, dass die Voruntersucher vom Knochenmark keine histologischen Schnitte sondern zytologische Ausstrichpräparate analysiert hatten Bei der Ausstrichtechnik lassen sich umschriebene Markprozesse aber nicht erfassen. So haben PIAZZA *et al* [25] die sich speziell mit den hämatologischen Veränderungen inokulierter Mäuse befassten nur zytologische Veränderungen der Hämopoese gesehen Sie fanden eine relative Verminderung der linksverschobenen Erythropoese und eine starke Zunahme der unreifen granulopoetischen Vorstufen. Letzteres deuteten PIAZZA *et al* als Reifungsstörung Nach unseren Ergeb-

nissen ist die Linksverschiebung jedoch ein Ausdruck der Wiederbe siedlung nekrotischer Markbezirke

Am nahezu regelmässigen Befall des Knochenmarkes bei der ex perimentellen Hepatitis der Maus kann kein Zweifel herrschen. Durch PIAZZA *et al* [25] waren bereits hohe Viruskonzentrationen in den hämopoetischen Organen wie Milz, lymphatisches System und Knochenmark nachgewiesen worden Wir fanden bei vielen Tieren ausser dem im Knochenmark synzytiale Riesenzellen als Ausdruck des zytopathogenen Effektes des MHV Virus (Abb 4) Diese Riesenzellen, von MALUCCI [22] in Peritonealdeckzell Kulturen als typisch beschrie ben kommen auch in den Lebern inokulierter Tiere vor

Unsere experimentellen Befunde legen eine Analogie zur menschlichen Hepatitis nahe. Sie lässt sich herleiten aus der beim Menschen – nach PITCHER und SPENCE [26] häufiger – vorkommenden Verände rungen des peripheren Blutbildes mit Leuko- und/oder Thrombopenie sowie diskreten Anämie mit verkürzter Erythrozytenlebenszeit und Anstieg der Retikulozyten [6] Die Analogie ist auch naheliegend wegen der gelegentlich auftretenden Kombination von Panmyelophthise und Hepatitis. Diese Kombination trat bei unseren Versuchen nur einmal auf. Daher ist anzunehmen dass es sich im Stadium der Virämie zwar um häufig auftretende aber reversible Veränderungen handelt, entsprechend der starken Regenerationspotenz des Knochen markes.

Genetische Schädigung der Hämopoese Wie schon im vorangegangenen Absatz diskutiert, sind Schädigungen der Hämopoese bei verschiede nen Viruserkrankungen bekannt. Der Erreger des Denguefiebers und das Masernvirus können schwere Chromosomenschädigungen hervor rufen Beim Masernvirus sind sogar völlige Zertrümmerungen der Chromosomen (Pulverisation) beschrieben worden [24] Eine Regenerationsstörung würde bei so schweren Zellschädigungen nicht über raschen. Wenn auch derartig schwere Schäden an Chromosomen des Menschen bei akuter Hepatitis nicht bekannt sind, so liegen doch Berichte über Chromosomenanomalien bei dieser Erkrankung vor [1] Ausserdem haben MELLA und LANG [23] gezeigt dass eine Mitosehemmung in Leukozytenkulturen eintrat, sobald man mit Serum von Hepatitiskranken inkubiert.

3 Eine *toxische Markschädigung* für den Menschen von verschiede nen Autoren diskutiert, lässt sich beim Fehlen jeglicher Korrelation zwischen Schwere der Leberfunktionsstörung und Häufigkeit der Kno-

chenmarkschädigung kaum vorstellen. Bei unserem Versuchsmodell sind toxische Schädigungen ursächlich nicht in Erwägung zu ziehen. Wie PIAZZA et al. [25] gezeigt haben, ist die Viruskonzentration im Knochenmark hoch. Dieser Befund passt zu den von uns nachgewiesenen Nekrosen, welche Folge eines Virusbefalles des Knochenmarkes sind. Die Annahme einer «toxischen» Markschädigung ist zumindest für das Modell der Mäusehepatitis überflüssig.

4. Antikörper gegen Blutzellen. Immunerkrankung. Die Frage, warum die Phthise als Folge des Knochenmarkbefalles auch tierexperimentell so selten ist, bleibt offen. Belege für einen ursächlich denkbaren Immunprozess fehlen uns. Für den Menschen hat BRICKERT [3] darauf hingewiesen, dass die Veränderungen «an die Verhältnisse bei immunologischen Zweitkrankheiten oder autoimmunologischen Störungen erinnern». Im Rahmen dieser Arbeit kann wegen des Fehlens entsprechender Befunde hierzu nicht Stellung genommen werden.

Zusammenfassung

Neunzig infantile CF_1-Mäuse wurden mit Mäusehepatitis-Virus (MHV) intraperitoneal infiziert, um mögliche Knochenmarkveränderungen zu analysieren. 15 der infizierten Mäuse starben innert 72 h. Die überlebenden 75 Tiere wurden bis zu 180 h später getötet. Im Knochenmark von Sternum und Femur fanden sich histologisch unterschiedlich ausgedehnte Nekrosen. Mit Fortdauer der Versuche und in grober Korrelation zu verschiedenen Verdünnungen nahmen die Nekrosen ab und die reparativen Vorgänge zu. Nach 180 h fanden sich praktisch normale Befunde. Bei einem Tier hatte sich eine unvollständige Phthise entwickelt, zwei zeigten Markhypoplasien. Die bisherige Annahme, die Schädigung blutbildender Zellen bei der experimentellen Hepatitis sei eine Reifungsstörung, kann nicht aufrecht erhalten werden.

Summary

90 infantile CF-mice had been inoculated intraperitoneally with murine hepatitis virus (MHV) in order to study reactions of the bone marrow. 15 of the infected animals died within 72 h, and the surviving 75 were sacrificed at intervals up to 180 h. Histological examination of the marrow of sternum and femur showed necroses of varying extension. With progressing time and in loose correlation to the different virus dilutions the necroses disappeared and reparative changes took place. Histological findings were virtually normal after 180 h. One animal developed an incomplete myelophthisis, two others hypoplastic marrow. The so far prevailing interpretation regarding the disturbance of blood forming cells during experimental hepatitis as a sign of maturation delay cannot be maintained.

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Adresse der Autoren: Prof. W. Hunstein, Drs. E. Pierson, B. Eggeling und N. Uhl, Medizinische Universitätsklinik, *D-34 Göttingen* (Deutschland)

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Laboratoire d'Anatomie Pathologique, Université de Liège, and Institute of Developmental Biology, Academy of Sciences of USSR, Moscow

Quantitative Study of the Hemopoietic Recovery after a Sublethal X Irradiation in the Mouse

J. Haot and N. F. Barakina

During the last decade, many researchs have been devoted to the problem of the small cell population of the marrow ('bone-marrow lymphocytes') and it has been suggested that this population contains the marrow stem cells [1, 2]. Particular attention has been drawn on the accumulation of small mononucleated cells in the bone-marrow of animals recovering from an irradiation [3, 4]. These cells, morphologically similar to the lymphocyte from which they can nevertheless be distinguished by some cytological characters, have received the name of 'transitional cells' [5, 6].

In a preceding work, we have studied the variations of this cell population in the mouse. Using for these investigations an electronic particle counter equipped with a plotter, we have shown that it was possible to differentiate in the femoral marrow between two populations of cells. The first is composed of small lymphoid cells in the volume range of 100–150 μm^3. The second one forms a peak at 220 μm^3 and contains cells of the granulocytic series [7, 8]. We have followed these two populations after an X-ray irradiation. The small cells rebound at the 11th day to a value higher than the normal while the population of larger cells recovers according to a biphasic pattern with a plateau at the 11th post-irradiation day. Combining the electronic counter method with a classical cytological study and the electron microscopy, we have shown [9] that between the 10th and the 13th post irradiation day, the group of small cells and medium sized 'lymphoid' cells was very heterogenous and was composed of at least 3 cell

types firstly the lymphocytes secondly some young granulocytic cells and thirdly a peculiar type of lymphoid-like cells, we called X-cells In the animals selected by means of the electronic counter for this experiment, the percentage of X-cells could be as high as 62% while the small lymphocytes seemed to represent no more than 14% of the total nucleated cells.

The purpose of the present work was to give some more precision on the variations of the marrow lymphoid cells during the recovery period. We decided to compare the cell populations that we could observe by means of the electronic counter and the populations calculated from the myelogram With the first method, we expect to obtain information about the population of small lymphoid cells (small lymphocytes.) The second method should allow the study of the total lymphoid like population including the X-cells. On the other hand, it appeared interesting to follow in the same animals the modifications of the other lymphoid organs thymus, lymph nodes and spleen.

Material and Method

R3 female mice 10 to 12 weeks old were X-irradiated with dose of 300 r (Stabilivolt Siemens apparatus, 190 kV 18 mA, 0.5 mm Cu filter 35 cmFD, output 210 r/min). Eight to 15 mice were killed by section of the cervical blood vessels each other day from the 2nd to the 20th post-irradiation day Eight to 10 additional animals were sacrificed on the 24th, 28th and 33rd post-irradiation days.

The blood was collected into glass tubes containing EDTA. 0.1 ml were hemolyzed in a cetrimide solution. 0.1 ml blood are dispersed in 23.7 ml balanced salt solution. After careful agitation, 1.2 ml hemolytic agent are added. This agent is prepared from stock solution of cetrimide at 20% acidified by acetic acid 4 parts plus 1 part acetic acid. The leukocyte counts were made with an electronic particle counter Hematocrit were measured. Smears were also prepared and stained with the May-Grünwald-Giemsa method. The number of granulocytes, lymphocytes and monocytes expressed in 10 per µl were calculated from the total number of leukocytes and their percentages on the smears.

The bone-marrow was extracted from the two femurs with 1 ml balanced salt solution (Hanks solution) and dispersed through a 24G needle. The suspension was kept in the refrigerator for 4 h. 0.1 ml were diluted in 25 ml cetrimide solution for the counting of the nucleated cells with the electronic counter 0.25 ml dispersed in 50 ml balanced salt solution served for the preparation of size distribution curves.

A B model Coulter Counter equipped with particle size distribution plotter was used. The aperture was 100 µm. The technical data were fixed in order to cover the volume range 0–300 µm³ Two populations of cells were calculated in the range 100–150 µm³ (small lymphocytes population) and 200–300 µm³ (large cell population composed of myeloid cells) The absolute number of cells in these populations was obtained from their percentages on the graphs and from the total number of marrow nucleated cells.

The remainder of the marrow suspension was centrifuged and 2 drops foetal calf serum were added to the cell sediment. Smears were prepared and stained by the May

GRÜNWALD-GIEMSA method. The cells were grouped in 5 major populations, i.e. young and intermediate granulocytic cells, mature granulocytic cells, erythroid cells, lymphoid cells, reticulo-endothelial cells. These populations were expressed in absolute number (percentages in the myelogram multiplied by the total number of marrow nucleated cells determined with the electronic counter)

Thymuses carefully dissected, blotted on filter paper were weighed. They were then delacerated and cut into pieces with pincers and scissors. After dispersion in 10 ml BSS, the suspension was passed through needles. The nucleated cells were counted after dilution in cetrimide solution part of the suspension was used for size distribution curves.

The spleen and lymph nodes were fixed in Bouin solution and embedded in paraffin slides were stained with hematoxyline-eosine for further histological study

Results

Figure 1 gives the results of the peripheral hematological values. The hematocrit is only slightly reduced between the 4th and the 12th day and is subnormal after this delay The blood leukocytes drop sharply at the second day they thereafter increase slowly till the 12th day The recovery is accelerated after the 14th day and a normal level is reached at the 24th day

The discrimination between the major cell types in the peripheral blood shows that the absolute number of granulocytes falls to a lower

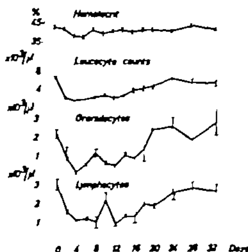


Fig 1 Effect of 500 irradiation on the hematocrit, the leukocyte counts and the absolute numbers of blood granulocytes and lymphocytes.



Fig. 4. Comparison of lymphoid ( ) and myeloid (b) populations studied by the electronic counter (------) and from the myelograms (———). Absolute number of cells from two femurs after a 500 irradiation.

it must be reminded that the cell number measured between 200 and 300 μm^3 (large cell population) represents only a fraction of the total myeloid population. After irradiation the curve relative to the large cell population detected with the Coulter (granulocytic cells) is in parallelism with the curve of the total granulocytic population determined by means of the myelograms. In both cases, the curves follow a biphasic pattern with a first recovery phase coming to a plateau around the 10th day and a second recovery phase starting after the 14th day.

The lymphoid population calculated from the smears that we shall call total population and the small lymphocyte population (from 100 to 150 μm^3) detected with the electronic counter show an overshoot about the 12th day. However the methods give results that are quantitatively very different. The overshoot is much more important when the population is calculated from counts on smears than when it is estimated from the volume distribution curves obtained with the counter.

In the thymus the total number of cells (fig. 5) drops sharply and remains very low till the 4th post irradiation day. The regeneration begins at the 6th day, is accelerated after the 8th day and normal levels are reached between the 12th and 14th days. After this delay we observed, as did many authors, a secondary atrophy. The vari

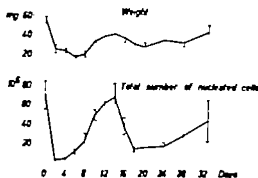


Fig 3. Variations of the weight and total cell number of the thymus after 500 irradiation.

ations of the thymus weight are very similar to these of the total cell number

The spleens are reduced in size and weight during the first post irradiation days. The recovery occurs at different rates for the hemopoietic (myelopoietic and erythropoietic) tissues and for the lymphoid tissue. At the 4th day blast cells and small clumps of erythrocytic cells are scattered through the organ The myeloid clumps are rare. All the hemopoietic foci are differentiated at the 8th day After this delay they grow bigger and are confluing From the 10th to the 20th post irradiation day the spleen the weight of which is far above the normal is mostly an hemopoietic organ After the 20th day there is a decrease of the erythropoietic and myelopoietic tissues with a slow return to normal level During the whole observation period, the megacaryocytic elements show only small variations.

The number and size of the lymphoid follicles are reduced during the first few days. The recovery seems to start between the 8th and the 10th day After the 12th day the regeneration is very fast and a return to a normal picture is seen between the 16th and the 20th day

In the lymph nodes, the destruction seems more severe than in the spleen and the recovery begins later on around the 12th day is slower and is only completed after the 20th day As already described by many authors the plasma cells are very numerous from the 6th to the 24th day Rare myeloid colonies are detected between day 12 and 20

Discussion

In the mouse exposed to a sublethal irradiation the two cell populations measured with an electronic counter regenerate according to very different patterns. The recovery of the large cell population appears to be biphasic with a plateau between day 12 and day 16 As we could expect the variations of this population which constitutes a representative sample of the total myeloid series are parallel to the results obtained with the myelogram The plateau around the 12th day corresponds to a lack of mature granulocytic cells (metamyelocytes and polymorphonuclear cells) the percentage of which sharply drops on the smears, while the young and intermediate cells are still increasing This is probably related to the delay required for the maturation of the dividing granulocytic compartment of the marrow the value of which was still very low a few days before.

The small cell population (small marrow lymphocytes) 100–150 μm^3 detected with the electronic counter recovers earlier with a small rebound at the 12th post irradiation day A more impressive overshoot is observed at the same delay for the total lymphoid population calculated from the myelograms. This difference is mostly due to the appearance in the marrow of lymphoid-like cells very similar to the marrow lymphocytes, with which they can easily be confused. Previous ultrastructural investigations have shown that these cells we called X-cells are however different from the small marrow lymphocytes [9]

The size of these cells is a little bigger than that of the lymphocytes on the volume distribution curves they are located between the small lymphocytes and the myeloid series. Therefore, they are situated in an overlap between the two populations detected in the marrow by the electronic counter For this reason it is impossible to measure accurately this cell group by this mean

We have tried to estimate indirectly the X-cells group by comparing the myelogram values and the volume distribution curves. From the electronic counter data, we have calculated the small lymphocyte population while on the smears we have determined the total lymphoid population which includes the X-cells besides the small lymphocytes and an insignificant proportion of small sized myeloid cells. The difference between the total lymphoid population and the small lymphoid population calculated with the electronic counter can then give a good measure of the X-cells group

Under our experimental conditions, it appears that the number of X-cells is very low at day 10, rises to a value of more than 6 millions at day 12 and recedes rapidly at day 14. What is the significance of this rapidly changing X-cells population? It is likely that these cells are the same as the lymphoid cells called 'transitional cells' by other authors which assume a relation between the transitional cells and other marrow elements [3, 4, 5, 6, 10]. The present results are in contradiction with this assumption. It appears from the comparison of the curve of the total number of nucleated cells in the marrow and of the variations of the X-cells population that the occurrence of the latter in the marrow coincides with a sharp increase of the total cell number and that their disappearance is parallel to a decrease of this number. If the X-cells were to transform into other cell lines, one would not expect such a decrease after the X-cell peak. On the other hand, a rapid increase of some other cell lines would be observed. No such variations happen and it seems necessary to find another explanation for the behaviour of the X-cells. In the absence of any sign of cell destruction in the marrow one must consider the possibility of an X-cell migration to other organs, for example the thymus, or the peripheral lymphoid organs. The evidence of a cellular exchange between the marrow and the thymus [11, 12] supports this hypothesis. There is indeed some parallelism between the development of the X-cells population in the marrow and the thymus regeneration. However, this could be purely coincidental. At the present, there is no direct proof for such an exchange as far as X-cells are concerned.

Summary

In the mouse exposed to a sublethal X-ray irradiation, the various cell populations of the bone marrow regenerate according to very different patterns. The erythroid series and the proliferative compartment of the myeloid series recover early between the 10th and 12th day while the maturation compartment is restored later on between the 14th and 24th day. The lymphoid population behaves quite differently and overshoots within 12 days. This rebound must be related to the appearance in the marrow of a peculiar population of lymphoid-like cells (X-cells or transitional cells). The relation between these lymphoid cells and other marrow elements is discussed.

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Authors' addresses: Dr. J. HAOT, Institut de Pathologie, Université de Liège, 1, rue des Bonnes Villes, 4000 Liège (Belgium); Dr. N. I. BARAKINA, Institute of Developmental Biology, Academy of Sciences of USSR, Moscow (USSR)

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Abteilung für Hämatologie, Zentrum der Inneren Medizin, Johann-Wolfgang-Goethe-Universität, Frankfurt/M. (Direktor: Prof. H. Martin)

Presence of Two Ph1 Chromosomes in Cells with 49 Clone from Patient in Blast Crisis of Granulocytic Leukaemia

M. H. Khan and H. Martin

Typical cases of chronic myeloid leukaemia (CML) are characterized by the presence of one Ph1 chromosome with altogether diploid number. Two Ph1 chromosomes like the aneuploidy have been reported in various cell lines of CML patients during the terminal phase of the disease. We report here a case of CML in blast crisis showing two Ph1 chromosomes in a cell-line with 49 chromosomes.

Case Report

A 57-year-old female was diagnosed as having CML in 1965. She was effectively treated with Busulphan till 17th of Feb. 69 when blast crisis occurred. The spleen was 12 cm and the liver 10 cm under the costal margin. Hb 11.2 g%, red cells 3.8 mill./mm³, platelets 122,000/mm³, white cells 129,000 with 64% myeloblasts. Bone marrow puncture at the time of admission revealed features of blastic transformation. Cytogenetic material was prepared directly from the same bone marrow specimen. On admission she was treated with 6-mercaptopurine (150 mg/daily) and prednisone (200 mg/daily). She died on the 27th Feb. 1969.

Results are shown in table I. A karyotype of bone marrow is shown in figure 1.

Discussion

New cell lines which may arise through mitotic non-disjunction from the 46 Ph1 positive CML cells by gradual neoplastic evolution are chronologically coincident in the great majority of the cases with the deterioration of clinical as well as haematological picture. In the

Table I. 47 available mitoses revealed Ph1 disomy. 27 mitoses were analysed

Cell No.	Chromosome No.	Extra chromosome	Missing chromosome
4	47	Ph1 Ph1	G
1	48	Ph1 Ph1 C, F	G D
1	48	Ph1 Ph1 C, F	G, E
21	49	Ph1 Ph1 C, F	G

case reported 49 cell line appears to be selected because of some particular advantage. 49 cell line in the terminal phase of the disease may be of interest in view of the other reported cases in which such a cell line with one Ph1 chromosome [3, 7] and two Ph1 chromosomes [1, 2, 4] have dominated. The clonal evolution seems to have evolved by a duplication of the Ph1 chromosome and acquisition of a C and a F extra chromosomes. Similar karyotypic pattern have been reported in two CML patients studied in blast crisis [1, 6]. It is generally agreed that additional chromosomal changes in the Ph1 positive CML cells would foreshadow an acute exacerbation. Ph1 disomy in diploid, hypodiploid and hyperdiploid cells may be exhibiting different trends of clonal evolution but as yet clinico-haematological differentiation cannot be revealed.

Ph1 anomaly is supposed to be present in the stem cells of erythroid, megakaryocytic and granulocytic precursors. The high frequency of Ph1 positive cells in the bone marrow of CML patient suggest that these cells with Ph1 anomaly are capable to become end cells. The chromosomal defect seems to be more deleterious to the granulocytic end cells. It could be speculated that cells with two Ph1 chromosomes as well as the aneuploid cells may not be capable to become end cells. Even if they do become end cells then it is quite reasonable to assume that such cells are functionally less capable than the CML cells having one Ph1 chromosome. In culture it has been demonstrated that the cells of CML have shorter life span than the corresponding normal cells [5]. The chromosomal defect appears to be responsible for the disturbed homeostasis of the hematopoietic tissue. The exaggerated activity of cambium layer producing increased number of precursor cells and the congregation of the precursor cells due to delayed differentiation and maturation will affect the environment incessantly resulting

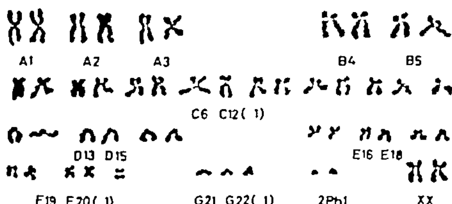


Fig.1 Karyotype of cell from bone marrow with 4? chromosomes.

in a vicious circle which will in turn enhance new mutations. The dominance of the established new clone or clones (e.g. Ph1 disomic cells) may implicate that the disease has irrevocably merged into its acute stage. It is rather early to comment on the differentiation and maturation capability of the aneuploid cell lines. Better understanding of the process of differentiation in the light of the present cytogenetic may be of some help in the circumvention of the neoplastic evolution.

We wish to acknowledge the expert technical assistance of Miss Christl Meckelnburg.

Summary

A hyperdiploid cell line with 49 chromosomes was dominant in the terminal phase of chronic myeloid leukaemia. Ph1 disomy and acquisition of C and F extra chromosomes as well as loss of G member was a consistent finding.

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Authors' address: Dr. med. Dr. rer. nat. M. H. Kuse and Prof. Dr. med. H. Martin, Zentrum der Inneren Medizin, Johann-Wolfgang-Goethe-Universität, L. R. [illegible] 14, D-Frankfurt/M. 70 (Germany)

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H. Stobbe und R. Sturzlitz: **Autoimmunerkrankungen in der Hämatologie.** Hämatologie und Bluttransfusionswesen. Vol. 3. Akademie-Verlag, Berlin 1969. 163 p. Preis DM 36.

In diesem Bändchen sind ausgewählte Beiträge und Diskussionen der IV. Hämatologentagung der DDR 1967 in Berlin zusammengestellt. Das Thema «Autoimmunerkrankungen in der Hämatologie» wird in erster Linie von der klinischen Seite her angegangen, wobei anhand von Patienten neben der Klinik auch Labormethoden und pathogenetische und ätiologische Gesichtspunkte besprochen werden. Auch die Therapie mit immunsuppressiven Medikamenten wird behandelt. Verschiedene Autoren betonen die noch ungewisse Indikationsstellung und die beim Menschen noch unbekannten Nebenwirkungen bei Langzeitbehandlung. Die 14 Aufsätze geben einen guten Überblick über den heutigen Stand des Wissens, speziell nützlich für den praktizierenden Internisten und Hämatologen. Für diese kann das Bändchen wärmstens empfohlen werden.

T. L. Vischer, Basel

R. Butler: **Isoantigenicity of Human Plasma Proteins.** Bibliotheca Haematologica No. 31. Karger, Basel/New York 1969. X + 107 p., 2 fig., 14 tab. Price sF/DM 33.–/US $ 7.90/66s.

With an already large and expanding volume of literature in this relatively new yet important field, this review about isoantigenicity of human plasma proteins must be welcomed. After giving the necessary definitions, the isoantigenic structures, mainly those on immunoglobulins and low-density lipoproteins, are reviewed. The corresponding antibodies, their possible mechanisms of formation in humans and their importance in clinical medicine are dealt with competently in the last chapter. The bibliography is abundant and up-to-date (December 1968). This book has been translated from the German language. Phrase constructions possible in German are often awkward to read in the translated English. However, this is a minor disadvantage considering the amount and quality of information presented.

T. L. Vischer, Basel

R. D. Eastham: **Klinische Hämatologie.** Springer, Berlin/Heidelberg/New York 1968. VII + 215 p. Preis DM 8.80/$ 2.20.

Das kleine, handliche – und erfreulicherweise sehr billige – Buch beschreitet nicht die ausgetretenen Wege der systematischen Lehrbücher, sondern versucht auf ganz andere Art dem Arzt oder Studenten eine Fülle von Information zu vermitteln. Dabei ist der Titel «Klinische Hämatologie» für deutschsprachige Leser sicher irreführend. Das Buch beschäftigt sich nämlich in erster Linie mit den verschiedenen, in der Hämatologie gebräuchlichen Laboratoriumsmethoden; was im deutschen Sprachgebrauch unter «Klinik» verstanden wird, ist dagegen kaum berücksichtigt. Im Laufe der letzten Jahre haben die Laboratoriumsuntersuchungen im Rahmen der klinischen Medizin aber immer mehr an Bedeutung gewonnen. Damit der Arzt dieses wertvolle Hilfsmittel voll ausnützen und aus den Ergebnissen die entsprechenden Schlüsse ziehen kann, muss er die Methoden richtig einsetzen und die gelieferten Resultate zu interpretieren wissen. Das Taschenbuch ist ganz darauf ausgerichtet, schnell und zuverlässig über die verschiedenen Labormethoden und ihre klinische Bedeutung zu informieren. Damit schliesst es zweifellos eine empfindliche Lücke im deutschen Schrifttum.

Neben Definitionen und kurzen Beschreibungen der Prinzipien der Methoden wird das Hauptgewicht auf die Interpretation der Resultate gelegt: wie sind die Normalwerte, was bedeuten pathologische Werte für die Klinik. Immer wieder sind auch pathophysiologische Grundlagen eingestreut. Der Hauptakzent liegt auf der morphologischen Hämatologie. Im ersten Kapitel werden Hämoglobin und verwandte Pigmente besprochen. Es folgen zwei

Abschnitte über Erythrozyten und Anämien, sodann zwei Kapitel über periphere weisse Blutzellen und Knochenmark. Mehr als ein Drittel des Buches wird vom Kapitel Blutungen, Blutgerinnung und Transfusion eingenommen. Der mehr immunologisch interessierte Leser wird allerdings nur vereinzelte – nicht immer ganz präzise – Hinweise finden, während die [illegible] Methoden sehr eingehend behandelt sind.

Die übersichtliche Gliederung und vorwiegend tabellarische Präsentation des Textes erlaubt eine rasche Orientierung und ist vor allem didaktisch von grossem Wert. Die knappen Literaturhinweise beziehen sich meist auf einschlägige, wenn auch ältere angelsächsische Arbeiten. Die Übersetzung kann im ganzen als sorgfältig bezeichnet werden. Der Bearbeiter hat einige nicht unwesentliche Ergänzungen und Korrekturen angebracht. Das Buch wird besonders dem jungen Arzt, aber auch dem hämatologisch interessierten Studenten ein grosser Helfer sein. Zweifellos wird auch das technische Hilfspersonal [illegible] Nutzen daraus ziehen. Wir wünschen dem für die praktische Tätigkeit sehr nützlichen Buch jedenfalls eine weite Verbreitung.

U. Bucher, *Bern*

E. Pedersen: **Cytogenetic Evolution in Chronic Myelogenous Leukaemia.** Munksgaard, Copenhagen, 1969. Relation of Chromosomes to Progression and Treatment of the Disease. XI + 121 p., Price: Dkr. 50.00.

This monograph reports a detailed and convincing study of the frequency and character of aneuploidy in the leukaemic cells of 50 patients with chronic myeloid leukaemia in relation to the clinical and haematological progression of the disease. After a preliminary survey of the cytogenetic background leading to the discovery of the Ph[1] chromosome and a comparison of the methods used in the study of chromosomes from haemopoietic cells, the author presents evidence confirming the hypothesis that not only granulocytic precursors but also erythroblasts and probably megakaryocytes in chronic myeloid leukaemia are Ph[1] positive. He then shows that patients in a late stage of the disease and those currently or recently receiving treatment have higher prevalences of Ph[1] positive hyperdiploid cells than patients in early stages of this illness. Hyperdiploidy also correlated with higher prevalence of myeloblasts and promyelocytes in peripheral blood, whereas when myelocytes were prevalent hyperdiploidy was infrequent.

A detailed analysis of the karyotype abnormalities at different stages of the disease suggests that the Ph[1] positive cell population progresses gradually to more and more abnormal karyotype as the disease evolves. There is some evidence that cytostatic treatment may encourage this progression. No such progression occurs in Ph[1] negative cells from the same patients, e.g. in phytohaemagglutinin-stimulated blood cultures, where at later stages of culture converted lymphocytes provide Ph[1] negative [illegible] The selection mechanism appears to operate especially in favour [illegible] and Ph[1] positive cells with [illegible] karyotype profile including extra chromosome [illegible] and absence of members of group E (17–18) and a [illegible] unique increase in chromosome numbers of leukaemic cells [illegible]

The implications of this elegant work in the wide field of [illegible] all concerned with chromosome studies in malignant [illegible] reading [illegible]

F[illegible]

E. R. G[illegible]: **Genetic Markers in Human Blood.** Blackwell, O[illegible]

This excellent book covers a wide field of laboratory and genetics. The author [illegible] R. G[illegible] is Research Professor of [illegible] of Washington School of Medicine and Head of Immunogenetics [illegible] Central Blood Bank, Seattle, Wa. In the first part of the volume

described which exhibit genetic polymorphism the immunoglobulins and their allotypes Gm and Inv haptoglobin, transferrin, the Gc system, the β-lipoprotein allotypes with the Ag and Lp systems, pseudocholinesterase plasma alkaline phosphatase, albumin variants, the protease inhibitor Pi system, ceruloplasmin, the -macroglobulin Xm system and the α_1-acid glycoprotein types. In the second part of the book genetic markers of blood cells are reviewed the red cell blood group antigens, the normal and abnormal hemoglobins and the enzyme variants of which the red cell acid phosphatase glucose-6-phosphate dehydrogenase 6-phosphogluconate dehydrogenase, phosphoglucomutase and adenylate kinase are most completely described. Smaller chapters deal with other enzyme markers such as lactate dehydrogenase, malate dehydrogenase, phosphohexose isomerase, carbonic anhydrase red cell esterases, peptidases, glutathione reductase, methemoglobin reductase, catalase, galactose-1-phosphate uridyl transferase and indophenol oxidase In the third and last part the basic principles of genetics and the contributions of blood genetic markers to studies of human biology are summarized.

The book provides review of the basic advances as well as instructions for the applications of these advances to the understanding of disease in rapidly growing field of modern hematology It can be recommended to laboratory investigators, clinical hematologists and blood bank serologists.

H. R. Marti, *Aarau*

Ch. A. Owen Jr E. W. Bowie, P. Didisheim and J. H. Thompson **The Diagnosis of Bleeding Disorders.** Little, Brown, Boston 1969 300 p. Price US $ 15.00.

This book in the series of laboratory medicine is concise presentation mainly written from the viewpoint of clinical pathologists. Consequently the methods for diagnosis of these disorders are well described. The introductory chapter covering the history of coagulation and hemostasis is welcome evaluation of the physiologists and clinicians who built the fundaments of this topic Their photographs with short biographic notes are given (among those Morawitz, Bizzozero, Fonio, Hammarsten, Alex. Schmidt Howell, Tocantins, Willebrand) A number of typical illustrative cases for the various haemorrhagic diseases are given. (The reading of the book would have been facilitated if these case histories had been printed in smaller type.) Each chapter has fairly comprehensive list of references.

The book will be of value as an introduction into the concept and methods of examination of blood coagulation and hemostasis which, as the authors rightly mention, are "in state of flux

G. Rosenow *New York, N.Y.*

Progress in Hematology Vol. 6. Editors E. B. Brown and C. V. Moore. Grune & Stratton, New York 1968. 380 p. Price US $ 19.75.

The new volume of this series contains critical reviews among others immunosuppression (Parker and Vavra), auto-immune hemolytic anemias (J. V. Dacie and Sr. Worlledge) prevention of Rh hemolytic disease (W. Pollack, J. Gorman and V. Freda) plasma fractions in the treatment of hemophilia and von Willebrand's disease (C. R. Rizza and R. Biggs) control of human hemoglobin synthesis and function in health and diseases (D. Weatherall and J. B. Clegg)

G. Rosenow *New York, N.Y.*

Varia

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International Atomic Energy Agency (IAEA)

IAEA Symposium on Dynamic Studies with Radioisotopes in Clinical Medicine and Research

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Organizers. International Atomic Energy Agency Kärntnerring 11–13, *1010 Vienna*, Austria. *Secretaries.* Dr T. Nagai and Dr E. H. Belcher, Medical Application Section.

The Symposium will be concerned with all applications of radioisotopes in clinical medicine and research which involve measurements of the temporal patterns of uptake, metabolism, clearance or excretion of administered radioactive materials. Topics to be covered include cardiac, gastrointestinal, hepatic, pulmonary renal and thyroid function studies, regional blood flow studies, calcium, copper iron, protein and vitamin B_{12} turnover studies and studies of red cell destruction. The Symposium will give emphasis to new instruments, techniques and methods of data analysis. Studies based on scintigraphic techniques will be excluded except in so far as they are concerned with dynamic situations.

Further information and forms to accompany abstracts of papers intended for presentation at the Symposium may be obtained from national authorities for atomic energy matters. Abstracts must be submitted through these authorities so as to reach to International Atomic Energy Agency before April 20, 1970

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Confecit G. Boehm, Basel

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